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Effect of nutritional status of dairy cows on the quality of ovarian follicles: an in vitro study

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A thesis

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by

Elbert Cabasag Gargar

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Master of Agricutural Science

Effect of nutritional status of dairy cows on the quality of ovarian follicles: an in vitro study

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Elbert Cabasag Gargar

The reproductive efficiency of dairy cattle around the world is declining as greater selection pressure for increased milk production is applied. In addition, nutrition also adversely affects reproductive performance. The purpose of this study was to investigate if the metabolic profile, that is to say, metabolism-associated serum analytes, bore any relation to the *in vitro* follicle growth and progression of ovarian cortex cultured in the presence of cows sera. Foetal calf serum (FCS) usually at a concentration of 10% in culture medium such as M199, forms the basis of most tissue culture systems including ovarian follicle cultures and has proved suitable for various species. In this study, cow serum (CS), sourced from a large number (17) of differently managed farms and/or animals to provide some variation in metabolic profile was used to replace FCS in the culture media. To date, no studies have been reported using sera other than foetal calf serum or calf serum for the *in vitro* culture of ovarian tissue. This study has shown that bovine ovarian follicle growth and progression can be supported *in vitro* by culture medium containing cow serum (CS). Morphologically the ovarian cortex remained viable after being cultured for a 10 day period in medium containing the gonadotropin follicle stimulating hormone (FSH) and supplemented with 10% CS. To assess the degree of follicular growth and progression occuring *in vitro*, quantitation criteria based on histological classification of follicles as either F_0 (primordial follicle- one layer of flattened somatic cells around the oocyte) or F_1 (primary follicle-a single layer of cuboidal granulosa cells around the oocyte) or F_2 (secondary follicle-surrounded by two or more complete layers of cuboidal granulosa cells) and their diameters as measured using image analysis software, were established. Follicles with diameters within diameter limits of 23-32, 41-53 and 55-70µm were classed as F_0, F_1 or F_2 respectively. To confirm if the follicle progression seen morphometrically *in vitro* was being driven by the genes known to regulate the process *in vivo*, the expression of Growth Differention Factor-9 (GDF-9) and Bone Morphogenetic Protein-15) (BMP-15) as represented by the relative concentrations of their respective mRNAs was determined by Quantitative Polymerase Chain

reaction (qPCR), was assessed. Relative to the reference gene (actin), GDF-9 and BMP-15 were expressed nearly 14x and 6x respectively in cultures demonstrating high follicle growth. In the absence of FSH in the cultures, gene expression levels were not elevated. Follicular growth and progression levels was negatively correlated with plasma concentrations of β-hydroxybutyric acid but not with other metabolic profile analytes or the fertility marker, antimüllerian hormone (AMH). This suggests that *in vitro* follicular growth in CS is influenced by the physiological/reproductive status of the cow and we conclude that an *in vitro* culture system is a practical approach for a sensitive follicle growth bioassay to assess the effect of the internal millieu with respect to fertility.

Keywords: bovine, *in vitro*, qPCR, FSH, *in vivo*, ovarian cortex, serum metabolites, follicles, metabolic profile, primordial follicles, primary follicles, secondary follicles.

Acknowledgements

The year when I was born, was a celebrated year in history. This was the period of time when America won the space race by sending first the man to the moon. At a young age I was fascinated by stories and remarkable world events. With all this great interest and enthusiasm, it came into the point that my parents called me “Apollo” and this line is my favourite:

“A small step of a man, a giant leap for mankind”

Neil Armstrong -1969-2012

Every single accomplishment, was only realised with voluminous amount of work and dozens of inspirational ideas. The people around me are the proof, they have pushed me and put more weight into the balance. My ever supportive wife Flora, sons Doughbert , Douglas and daughter Dawn. I am also grateful to my supervisor Dr. Graham Kay and my Associate Supervisor Dr. Rob McFarlane; you did add multiple cubits to my stature, and the knowledge gained will surely better prepare me to face the more challenging future ahead with additional strength. I know this would be of great value ‘til senescence. Due thanks also to Joshua Phillips for his brilliant laboratory technical support and likewise to Dr. Jim Gibbs for supplying the cow blood. Lastly to all the nice people who worked behind the scences to help made this work possible. Much appreciation and thanks to the management and staff of the Ashburton Meat Processors who provided cow ovaries for this study from start to finish without a hitch; from ovary count to the timing of pick-up, everything went like clockwork.

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Chapter 1

Introduction

Dairy cow fertility in New Zealand and the rest of the world has been seen to be declining (Lucy, 2001; Royal *et al.*, 2000). The decline in fertility has been dramatic and worrying. In the US dairy industry, conception to the first mating has dropped from 55% to about 35% over a 20-yr period (1976-1999)(Washburn *et al.*, 2002). Data from the New Zealand Livestock Improvement Corporation show that the average number of inseminations per cow to achieve a pregnancy over the last 20 seasons has risen from 1.26 in 1990/91 to 1.32 in 2009/10 and survey data have indicated that the routinely achievable conception rates to the first mating of 65% decades ago had slipped to 55% or less with the modern high producing type of cows (Macmillan *et al.*, 1984; Xu & Burton, 2003). Breed preference change from Jerseys to Friesians has been implicated in the general loss of fertility as Friesians take longer to resume oestrus than their counterpart breeds and display greater sensitivity to a change in body condition (Burke *et al.*, 1995; McDougall *et al.*, 1995; Xu & Burton, 2003). This declining fertility in dairy cows is a concern to the world dairy industry as well as the NZ dairy industry.

The New Zealand Holstein-Friesian line of cows (Harris & Kolver, 2001) was established from animals imported from West Coast of the US until 1925. Importation was resumed again in 1960. Further selection was done for potential increase in milk production by incorporating North American Holstein-Friesian genetics by means of imported frozen semen. North American Holstein-Friesian genetics have had a significant influence on NZ dairy populations, increasing genetic contribution from 2 to 38% in 20 years. Concurrent with this increasing North American influence, milk yield increased by more than 16% and milk solids (MS) by 23% on pasture-based systems. However with the selection for milk production, fertility in both NZ type cows and North American type cows has declined (MacDonald *et al.*, 2008).

North American-derived Holstein cows brought a dramatic increase in milk production (Washburn *et al.*, 2002), were heavier and produced more milk volume and protein yield (Harris & Kolver, 2001). Data from NZ Livestock Improvement Corporation (LIC, 2010) showed that total milk processed from seasons 1980/81 to 2009/10 increased from 491×10^6 Kg MS to 1.438×10^9 Kg MS but production varies across NZ. The impact of milk production on reproduction has been expressed in various ways: each 1,000 kg increase in milk yield per cow was associated with a decrease of 3.2 to 6% in pregnancy rate, a decrease of 4.4 to 7.6% in the number of cycling cows, and with a 4.6 to 8% increase in the incidence of inactive ovaries (López-Gatius *et al.*, 2002). In another study it was

concluded that over the past 30 to 50 years, intensive selection for milk yield traits has led to a reduction in first-service pregnancy rate from 70% to 40% (Dobson *et al.*, 2008).

The vast majority of dairy cows in NZ are artificially inseminated (over 2.5 million dairy cows each year) as part of the LIC premier Sire program. During the compact 6 week breeding season running from mid-October, semen from different bulls is distributed daily across the country to AI technicians, providing herd owners with high quality genetics from a range of bulls, resulting in broad terms, in a national herd with relatively uniform genetics of North American origin.

Despite the distribution of genetics and the widespread use of the Dairy NZ 'Incalf' management system, milk production varies across New Zealand. Assuming equivalent standards of management, the factor most likely responsible for this regional variation is nutrition. The poorer the nutrition that cows get, the poorer the milk production. In the statistical analysis of the Dairy industry (LIC, 2010), the average yield of milk solids per cow was highest in North Canterbury (382kg/cow) and lowest on the East Coast of the North Island (238kg/cow) while the lowest in the South Island was the West Coast region (308kg/cow) reflecting regional differences in pasture qualities. The differing of pasture quality across regions is shown in the stocking rates across the country with North Canterbury the highest at 3.3 cows/ha and the West Coast of the South Island being the lowest at 2.2 cow /ha.

It is well known that poor nutrition has a negative impact on reproduction efficiency (Cardoso, *et al*, 2013) and it is likely that the varying nutritional conditions across the country play a role in the fertility of the New Zealand dairy cow. The NZ dairy industry is almost uniquely exclusively pasture-based and the seasonal changes in the pastures dictate the similarly unique seasonal breeding pattern. Seasonal calving in the NZ farming system must be in synchrony with grass growth (Dillon *et al.*, 2006; Walsh *et al.*, 2011) and grass production must be as its peak to sustain animal daily requirements for optimum productivity. Conversely, pasture resources are limiting during the winter season where animal feed requirements are considerably altered as all cows should be pregnant at this time of the year to fit into the spring calving pattern.

The average high producing cow needs approximately 17-18% crude protein (CP) in the dry matter (DM) they consume, likewise it has been reported that diet components and their relative proportions significantly influence animal production(Muller, 1993; NRC, 1989). Further, while well-managed pastures provide a high quality feed, they do have a number of deficiencies which limit productivity (Ulyatt & Waghorn, 1993). Moreover, pasture quality changes with season (Moller *et al.*, 1996; Verkerk, 2003) and the high CP levels that are found mainly in spring and autumn pasture are likely to create a "protein penalty" since the cow must use energy to clear urea from the

bloodstream, which could otherwise be used for milk synthesis (Beever, 1993; Danfaer *et al.*, 1980; Satter *et al.*, 1992). Therefore, pasture conservation is practiced to supplement feed deficit requirements to buffer animal feed intake with less guarantee on its nutritional components.

The pasture-based seasonal systems dictate that animals be bred over a very short period compared to many overseas countries. Data have shown that more than 90% of all NZ dairy farms operate spring calving, seasonal milk supply, pasture-based systems. The management of grazed pasture therefore requires a compromise between quality and quantity, and the level of accumulation of pasture is governed by appropriate stocking rate, grazing rotation length and stringent rules for conservation (Clark, 2002). Forage pasture is a staple to NZ dairy cows and the data in Economic Farm Survey identified that the dry matter intake of the average NZ dairy cows was made up from 88.5% grazed pasture, 5.5% pasture silage, 3.0 % maize silage, 2.0% purchased grazing and 1.0% other supplement (Anonymous, 2000-2001).

Genetic correlation between milk yield and fertility could be caused by pleiotropic gene effects (Veerkamp *et al.*, 2003) that is, variation in genes that increase milk secretion in the udder may simultaneously affect fertility through direct effects on high yield, a greater negative energy balance (NEB) when feed cannot match the additional energy demands, and could lead to subsequent poorer oestrus behaviour (Stevenson, 2001). In fact cows go into major NEB for the 40 days or so after calving. If cows are to calve every 365 days, this period of NEB coincides with the period during which ovarian follicles are growing and progressing in advance of the animals conceiving again (Figure 1.1). Transition nutrition (early lactation) post- calving in dairy cows was found to alter the cow's metabolic priorities for available nutrients from milk production and the mobilisation of tissue reserves. In this situation, if the dairy cow cannot adapt quickly to these challenges, this will result in a substantial impact on milk synthesis during early lactation which places the transition dairy cow in a state of severe NEB where energy output exceeds energy intake (Grummer, 1995), that can affect the duration of a dominant follicle, the number of follicle waves per cycle and have an indirect effect on conception rates (Crowe, 2008)

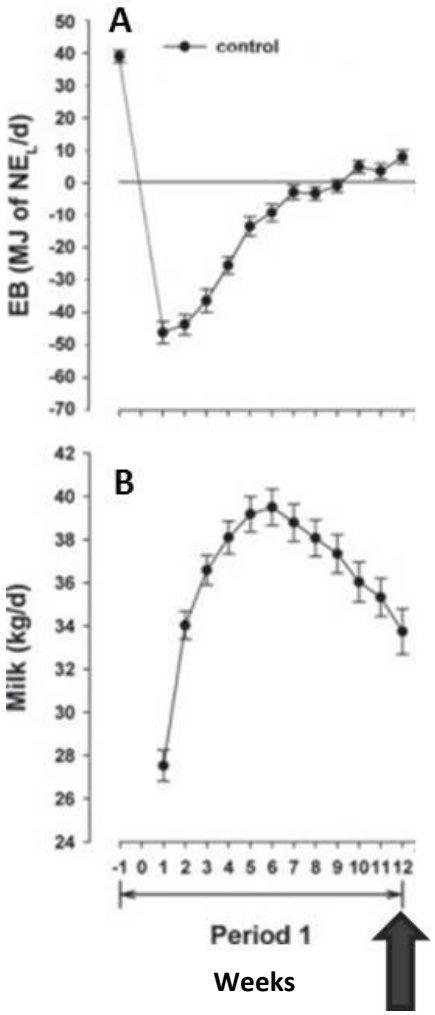

Figure 1.1 Diagram showing the relationship between calving (0 on the x axis), energy balance (A), milk yield (B) and artificial insemination (arrow). To fit an intercalving period of 365 days, inseminations take place shortly after the animals return from approximately 9 weeks of NEB. Adapted from the study of Gross *et al*., (2011).

NZ has a unique dairy farming system, implementing strict seasonal milking, and therefore strict seasonal reproduction management, and can be summarised as artificial insemination in November/December; pregnancy – November/July; calving – August/September; lactation - August/May with peak lactation about October. To be successful, the focus of the reproductive management is a submission rate target of 90- 95% of a herd submitted for insemination during the first 3 weeks of the breeding period, with conception rates to insemination of 60% or better (Smith *et al.*, 2001). A delay of more than 42 days has significant disadvantage during the subsequent breeding season because of their shorter postpartum interval and reduced fertility, that is determined by the knowledge of the seasonal pasture growth patterns (Verkerk, 2003).

Under sub-optimal nutrition conditions or NEB, cattle change their metabolism and there is utilisation of fat reserves, breakdown of muscle protein and a reduction in liver protein synthesis. This is reflected in changes in the concentrations of some plasma metabolites. As triglycerides are

broken down, fatty acids are released and the concentration of these increases in the blood and are quantified as non-esterified fatty acids (NEFA). As fatty acids are metabolised via β-oxidation, various ketone bodies are produced, one of which is β-hydroxybutyric acid (BHBA). Under conditions which result in consumption of body reserves, both NEFA and BHBA concentrations in plasma increase. Likewise, during this time when body reserves are being used, serum albumin concentrations decrease. Rowlands *et al*., (1980) found that serum albumin decreased in dairy cows at or close to calving and concentrations did not return to pre-calving values until 2 or 3 months later. Decreased blood glucose concentrations after calving have been observed by a number of authors (McClure, 1977; Rowlands *et al.*, 1980) and prolonged decreases in glucose appear to occur more commonly in high-yielding cows, which are frequently in negative energy balance. Consequently, low plasma glucose is reflecting low carbohydrates intake; liver protein synthesis is suppressed and subsequently plasma albumin low. Analysis of these and other blood components has been termed the metabolic profile and serves as a biomarker for nutrition status.

There is high correlation between NEFA concentrations and body condition score (BCS) which is an external estimate of the subcutaneous fat and muscle coverage of an animal (Prodanovic *et al.*, 2012; Stengarde *et al.*, 2008). With respect to reproduction, ideally dairy cows should calve with a BCS of 2.75–3.0 (on a scale 1-5) and not lose more than 0.5 of a BCS unit between calving and first service (Mulligan *et al.*, 2006; Overton & Waldron, 2004). Cows that lose excessive body condition (≥1.0 BCS unit) have a longer postpartum interval to first ovulation. Low BCS with severe NEB during early lactation period suppresses pulsatile LH secretion, reduces ovarian responsiveness to LH stimulation and also reduces the functional competence of the follicle characterised by reduced oestradiol production (Diskin *et al.*, 2003) and ultimately results in delayed ovulation (Butler, 2003). This has been associated with metabolic-related diseases, decreased fertility and increased culling rates (Fourichon *et al.*, 1999; Gröhn & Rajala-Schultz, 2000; Hayirli *et al.*, 2002; López-Gatius *et al.*, 2002; Lucy, 2001; Maizon *et al.*, 2004; Morrow, 1976). Changes in metabolic profile analytes varies with the type of NEB, suggesting there is a difference between NEB brought about by feed restriction as opposed to lactation-induced NEB (Gross *et al.*, 2011; Prodanovic *et al.*, 2012).

Gametogenesis in cows and bulls is distinctly different and the extended period developing eggs find themselves in may contribute to their vulnerability and reduced viability. In bulls, the process of spermatogenesis (spermatogonium to mature sperm) takes about 50 days. If during this time, the bull is for example exposed to a fever, sperm may be abnormal and compromised but as spermatogenesis is a continuous process, the compromised sperm are soon replaced and sperm quality returns to normal. In contrast, oogenesis in females commences during foetal life (Erickson, 1966b) and the pool of primordial follicles formed at this stage is not added to, rather, the pool

reduces in size over the rest of the animal's fertile life (Erickson, 1966a). Thus any insult, nutritional or otherwise, suffered by the cow at any stage of her life, is likewise experienced by the ovarian follicles. The number of follicles remaining in an ovary is referred to as the ovarian reserve and is positively associated with fertility and serum concentrations of the hormone Anti-Müllerian hormone (AMH) show a positive correlation with ovarian reserve (Ireland *et al.*, 2010) and are used as a fertility marker.

There is evidence that nutritional insults have an impact on BCS coupled with severe NEB resulting in reduced ovarian responsiveness to LH stimulation and also reduced functional competence of the follicle characterised by reduced oestradiol production (Diskin *et al.*, 2006) and results in delayed ovulation (Butler, 2003). There is also an indication that plasma concentrations of progesterone at the time of emergence of the second follicle wave may be lower and a consequence of extended periods of negative energy balance in early lactation. Failure to maintain adequate body condition in the New Zealand pasture-based feeding system has been seen to result in failures in ovarian follicle development and pregnancy non-recognition which have a detrimental effect on fertility (Verkerk, 2003). These findings support the hypothesis that exposure of ovarian oocytes to unfavourable physiological events during follicle development may result in the short term effect of ovulation of defective oocytes up to 3 months after the insult (Britt, 1992; Fair, 2010) with possible long term effects also (Ireland *et al.*, 2011).

The bovine ovarian follicle is difficult to study *in vivo* but maintaining ovarian tissue *in vitro* allows some experimentation, even to the point of producing live offspring. Oocytes have been found to progress *in vitro* as normal ova that have been produced in mice (dela Pena *et al.*, 2002; Eppig & Schroeder, 1989; Hagesawa *et al.*, 2006; Smitz & Cortvrindt, 2002; Spears *et al.*, 1994) and offspring have even derived from oocytes from primordial follicles (Eppig & O'Brein, 1996; O'Brien *et al.*, 2003), while in cattle oocytes *in vitro* culture for the latter half of their life were observed (Hirao *et al.*, 2004; Yamamoto *et al.*, 1999).

The hypothesis being investigated in this study is that subtle fertility-related changes in the blood of dairy cows, brought about by variation in the nutritional status, will impact on the growth and progression of ovarian follicles in an *in vitro* culture system. Specifically, this study aims to:

1. Establish a novel culture system using dairy cow serum.
2. Develop a means of quantifying follicles classes.
3. Test the influence of dairy cow serum on follicle growth and progression.

Chapter 2

Materials and Methods

2.1 Bovine ovary collection and processing

Batches of ovaries (n=18-22) from unknown adult cows were obtained weekly from a local abattoir (Ashburton Meat Processors, New Zealand) over the period January to June 2012. Generally, the cows were about 2 years old. Nothing was known about their reproductive history. The ovaries were refrigerated after collection and then placed in a flask containing melting crushed ice and transported to the tissue culture laboratory at Lincoln University for processing. The elapsed time between slaughter and placing the tissue in the culture was less than 5h.

The ovaries were washed free of blood and the surrounding fat and fibrous tissue were trimmed off. The ovaries were individually weighed before further washing in water with a few drops of surgical soap (Biodine, Vetpharm, Auckland, NZ) and thorough rinsing under running tap water. The cleaned ovaries were transferred to a sterile closed container for further processing in a laminar flow hood. In the laminar flow hood, 1mm thick strips of ovarian cortex were shaved off each ovary using a microtome blade anchored on 25x75x1mm glass slides secured with small paper clips (Fig.1A). The cortical strips were transferred to a 50 ml tube containing Dulbecco's PBS (Sigma-Aldrich Co., St. Louis, MO, USA) containing 100units penicillin/ml and 100µg streptomycin/ml (GIBCO®, Invitrogen Corporation, Auckland, NZ; DPBS). Using a set of razor blades spaced 3mm apart, the ovarian cortex pieces were first cut into strips and then by rotating through 90^{o} into 3x3mm squares (Fig.1B). The squares from all ovaries were pooled in a petri dish (Nunclon™, Cat. No. 172958) (Fig.1C), and washed three times in the DPBS before being placed in the culture (Fig.1D).

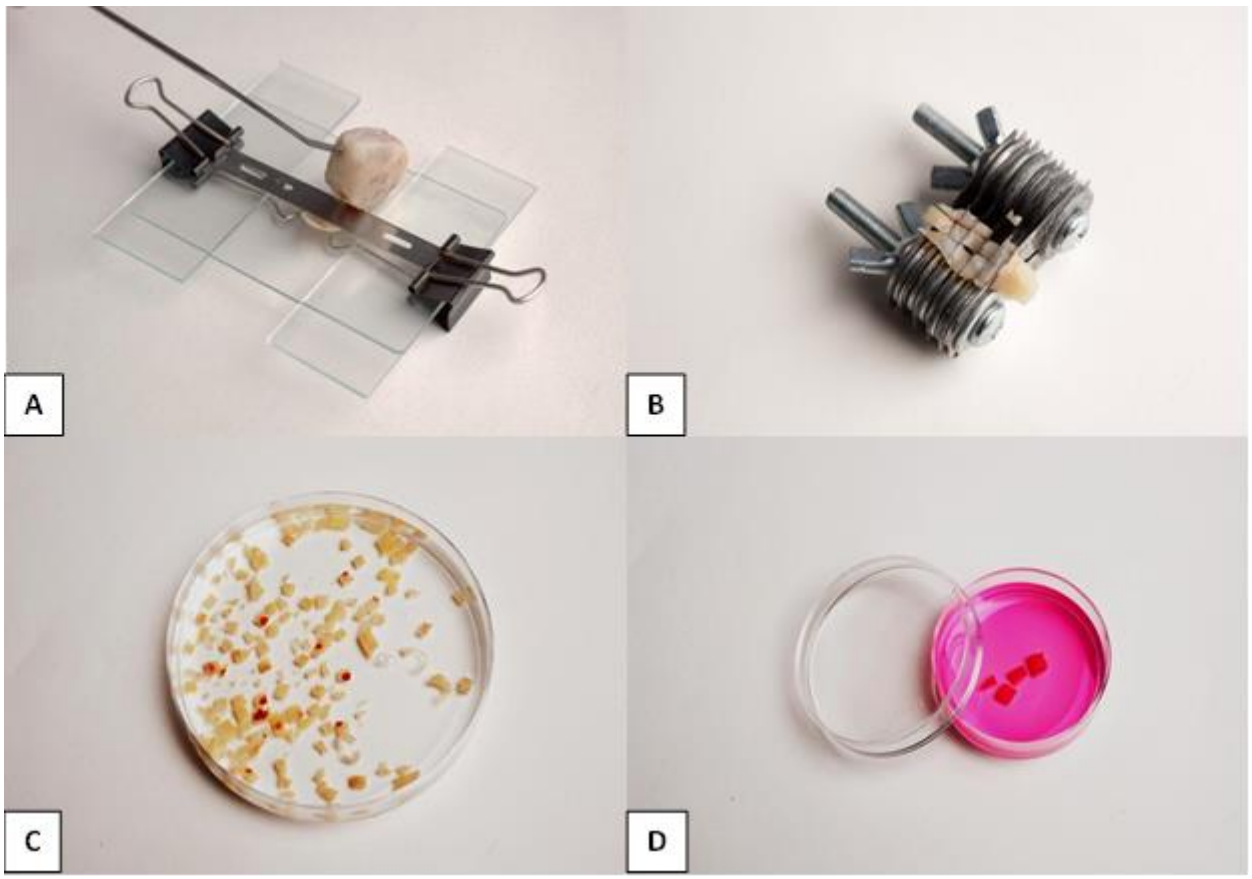

Figure 2.1 Preparation of cortical tissue for culture. A. Microtome blade fastened to glass slides used to shave 1mm bovine cortical strips. B. Improvised blade set to cut 3x3mm squares. C. Pool of cortical squares in 100x20 petri dish in DPBS + antibiotics. Dark red cortical squares contained corpus luteal tissue and were not selected for culture. D. Cortical squares in 35x10mm petri dish ready for incubation.

2.2 Culture medium

Cortical squares were cultured as described by Wandji *et al*., (1996) in medium M199 (Sigma-Aldrich, Co. St. Louis, MO, USA) containing 10% serum (see below for details) and 100 units penicillin and 100µg/ml of streptomycin (GIBCO®, Invitrogen Corporation, Auckland, NZ). To eliminate the effect of progesterone from active corpus luteal tissue, all cortical culture medium included 1µg prostaglandin $F_{2\alpha}$/ml (Ovuprost®- Cloprostenol (Sodium) 250µg/ml, BOMAC Laboratories Ltd., Manukau, New Zealand; $PGF_{2\alpha}$). In addition, culture media were supplemented with pregnant mare gonadotropin (SIGMA®-Sigma Chemical, Co., St Louis, MO, USA; PMSG) or porcine follicle stimulating hormone (FOLLTROPIN®-V, Bomac Laboratories, Ltd. Manukau, New Zealand; FSH) at various concentrations (see individual experiments for details). To minimise the variation between medium preparations, sufficient medium was made up at the commencement of an experiment to include all the ½ media volume changes.

The serum used to supplement the M199 was either bovine foetal calf serum (Medica Ltd, Auckland, New Zealand) or cow serum. The sera representing differing nutritional situations were generated by

pooling serum from groups of dairy cows from different South Island locations (West Coast, Southland, Canterbury) at different times of the year (September – February) to cover the end of winter, spring and summer periods in areas known to likely have poor (West Coast), moderate(Southland) and good pastures (Canterbury). Serum samples from 15-20 cows at each period/location were combined to yield 17 different serum pools. Coccygeal vein whole blood samples (10ml) were collected and were stored overnight at 4^{o}C to allow for clotting then centrifuged at 4,000rpm for 15 minutes for separation of serum. The serum pools were aliquoted and stored at -20^{o}C.

2.3 Characterisation of the cow sera

2.3.1 Metabolite analysis

Aliquotes (5ml) of each serum pool including the foetal calf serum (FCS) were assayed for albumin, β-hydroxybutyric acid (BHBA), glucose and non-esterified fatty acid (NEFA) by Gribbles Veterinary Pathology, Christchurch, New Zealand using the Roche Hitachi Modular P 800 analyser (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Albumin was quantified in a colorimetric assay using bromocresol green binding (Cobas-Roche, West Sussex, England). Glucose was quantified by generation of NADPH (Cobas-Roche, West Sussex, England). β-hydroxybutyric acid was quantified using a kinetic enzymatic method in which $NAD^{+} \rightarrow$ NADH (Randox). Enzymatic conversion of NEFA to H_2O_2 and subsequent formation of blue purple pigment was used to quantify serum NEFA (Wako Diagnostics, VA, USA).

2.3.2 Anti-müllerian hormone assay

Serum concentrations were determined using anti-müllerian hormone (AMH) Gen II ELISA (Beckman, Coulter, Inc., USA, Cat. No. A79766/A79765) kit. This is a sandwich type assay employing capture antibodies raised against purified bovine AMH. The secondary antibody is linked to horse radish peroxidase via biotin/ streptavidin. The calibration curve was created over the range 0 to 20ng AMH/ml. All samples and standards were assayed in duplicate and the absorbance at 450 nm was read in a FLUOstar Omega multidetection microplate reader (BMG LABTECH, Germany). The assay was carried out according to the manufacturer's instructions.

The standard curve was linear with an r^2=0.9931 (Fig. 2.2).

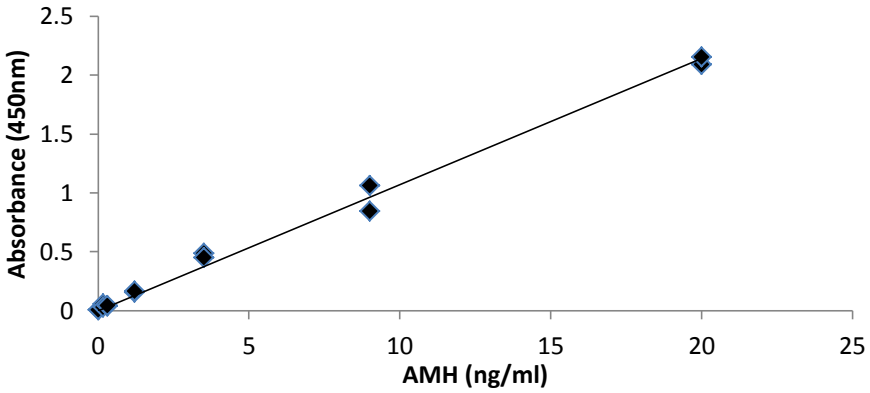

Figure 2.2 The AMH ELISA standard curve, $y=0.1069x$, $r^2= 0.9931$.

2.4 Ovarian cortex culture conditions

Three to five ovarian cortical squares as described above were placed in a 35x10mm petri dish in duplicate with 2ml of M199 culture medium containing serum, antibiotics, $PGF_{2\alpha}$ and gonadotropins and cultured for up to 10 days in 5% CO_2 and 100% humidity at 37^{o}C. The CO_2 concentration in the incubator was validated by gas analyser (FYRITE®, Bacharach, Inc. PA, USA). Half medium changes were done every 2-3 days. At the end of the culture period (see individual experiments) the cultures were terminated by fixing the cortical squares in formyl saline (10% formaldehyde in 0.15M NaCl solution) for cryostat sectioning and follicle morphometric analysis.

2.5 Morphological evaluation and histology

Fixed cortical squares (3-5) were placed on strips of paper (Fig. 2.3A), transferred onto the cryostat chuck (Fig. 2.3B), covered with several drops of optimal cutting temperature compound (OCT) freezing medium (Jung, Leica Microsystems, GmbH, Wetzlar, Germany) and allowed to freeze at approximately -20^{o}C (Fig.2.3C). Frozen tissues were sectioned at a thickness of 6µm (Fig.2.3D) on a cryostat (Leica CM1100, Leica Microsystems, GmbH, Wetzlar, Germany). Every 8th section was mounted and 4-5 sections were mounted per slide on a poly-lysine coated slide (25x 75mmx1mm, Labserv, New Zealand, Cat. No. LB4951+) (Fig. 2.4A) and dried overnight at room temperature.

Figure 2.3 Sectioning cultured ovarian cortex to assess follicle growth and progression. A. Fixed cortical squares from one culture dish lined-up on a paper strip ready to load onto the chuck. B. Chuck loaded with cortical squares. C. Chuck with frozen optimum cutting tissue (OCT) ready for sectioning. D. A 6µm tissue section coming off the block.

The sections were hydrated in water, stained with haematoxylin (Harris, VWR International Ltd, England) and eosin and then dehydrated in an aqueous ethanol series (70%, 90%, 100% I and 100%II, xylene I, xylene II, 1 minute each) and cover-slipped with DPX mounting medium (BDH Chemicals Ltd, Poole, England) (Fig.2.4).

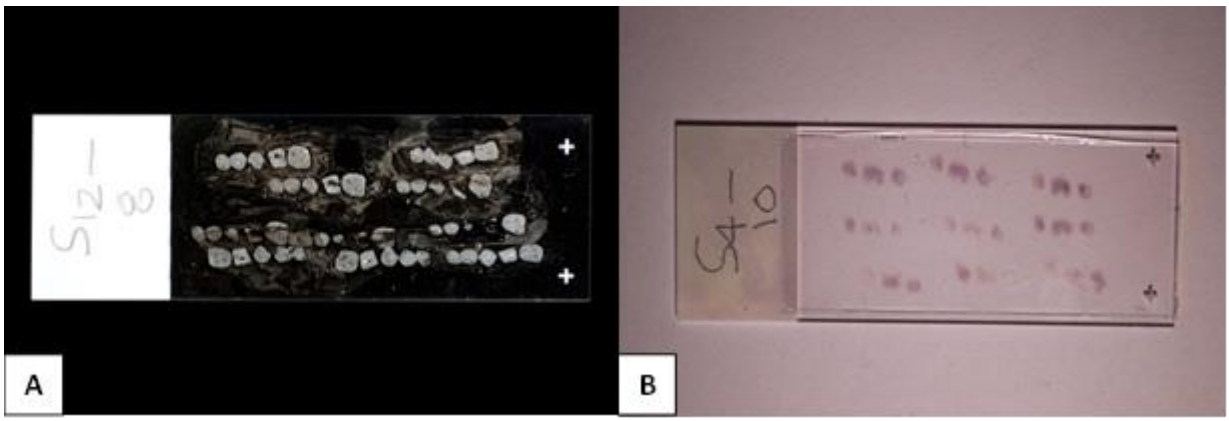

Figure 2.4 Follicle growth and development assessment in serial sections of multiple cortical squares of tissue. Microscope slides (75x22mmx1mm) with 6 µm sectioned cortical squares A. Unstained, B. Stained with Haematoxylin & Eosin.

Stained cortical sections were viewed at 200x magnification (Nikon Microscope Eclipse 50i). Primordial (F_0), primary (F_1) and secondary (F_2) follicles were in the first instance identified by their follicle cell layers. To facilitate the routine classification of large numbers of follicles as F_0, F_1 and F_2, 80 of each were identified and their equatorial diameters were determined from cross sectional area measurements using NIS-Elements Software (Nikon Instruments Inc., Melville, NY, USA) and means and standard deviations determined. Software used to determine area measurements was validated using a known area (Improved Neubauer counting chamber Hawksley, Sussex UK).

2.6 Minimisation of variation between cultures

Several steps were taken to minimise the variation between cultures. These included:

1. Making up batches of culture medium sufficient for each experiment.

2. Monitoring homogeneity of ovaries by weight recording ovary size from week to week.

3. Monitoring incubator performance (temperature, water level, humidity and $\%CO_2$, by both colour of pH indicator in the culture medium [phenol red]) and Fyrite analysis.

4. A large pool of cortical squares (approximately 200 or more) was generated for each experiment from about 20 ovaries.

2.7 Expression levels of GDF-9 and BMP-15 relative to Actin

The level of expression of a particular gene in living tissue can be estimated by quantifying the number of copies of the specific associated mRNA. To achieve this, RNA extracted from the tissue is first reverse transcribed to create DNA, known as cDNA, from the RNA. By using specific primers for the genes of interest in a PCR reaction and monitoring in real time the amount of DNA product (qPCR), an estimate of the original amount of specific mRNA can be obtained. The level of expression of a house keeping or reference gene is concurrently estimated and the results expressed as a gene of interest: reference gene ratio. Lockey *et al.*, (1998); Steuerwald *et al.*, (1999); Kubista *et al.*, (2006) found that qPCR has substantial advantages in quantifying low target copy numbers from limited amounts of tissue or identifying minor changes in mRNA or microRNA expression levels in samples with low RNA concentrations or from single cells analysis.

2.7.1 Reverse transcription

Tissue samples (cortical square pieces) at the end of 10 days in culture were used for gene expression studies. To minimise RNA damage, samples were directly snap frozen in liquid nitrogen and kept at -80^{o}C freezer until use. Tissue sample grinding was performed using a mortar and pestle/liquid nitrogen), RNA was extracted in Trizol reagent (Invitrogen Co, Auckland, New Zealand) and chloroform, precipitated with isopropanol and re-suspended in RNase free water (See appendix 1 for details). All primers (Table 2.1) were commercially synthesized (Integrated DNA Technologies, Belgium). PCR products were analysed on agarose gel electrophoresis. The purity of each RNA extraction was checked by determining the A260/A280 ratio and end-point PCR was set up using DNA polymerase and RNA as template to check the presence of contaminating DNA. Total RNA was reverse transcribed in a thermocycler under the following conditions: 37°C for 15min (Reverse transcription) 85°C for 5sec (Inactivation of the reverse transcriptase with heat treatment) then held at 10°C indefinitely.

2.7.2 qPCR

The qPCR analyses for the gene expression of bt GDF-9, bt BMP-15 and bt Actin in cultured cortical square tissues were carried out by SYBR assay as previously reported by (Hayashi *et al.*, 2010; Hosoe *et al.*, 2011; Ushizawa *et al.*, 2007). The following were the thermal cycling conditions and included initial sample incubation at 50^{o} for 2 min and 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 15 sec and at 60^{o}C for 1 min. The cycle threshold values (ΔCt) indicated the quantity of the target gene in each sample, and the sequence of the target gene was determined in real time using an Eco™ Real-

Time PCR System Ver. 4.0, Illumina® (San Diego, CA, USA). Standard curves were generated for each gene by serial dilution of the cDNAs (bt BMP-15, bt GDF-9, and bt Actin) to quantify the amplified products. Precision pipetting of reagents were carried out by ep*Motion*® 5070 with integrated PC and epBlue™ Version 10 (Hamburg, Germany). Each reaction was carried out in duplicate. The relative expression level of each target gene was calculated by the relative standard curve and ΔΔCt methods using EcoStudy software, Illumina® (San Diego, CA, USA).

Table 2.1 Reverse transcription and qPCR primer sequences and fragment sizes.

Process	Gene	Accession number	Amplicon size (bases)	Primer sequence
RT	BMP-15	AY572412	377	F: 5'-CAAGCAGGCAGTATTGCATCTGAA-3'
				R: 5'- TCACCTACATGTGCAGGACTGGGC-3'
	GDF-9	AB058416	401	F: 5'- GAAGCTGCTGAGGGTGTAAGATT-3'
				R: 5'-AAGCAATTGAGCCATCAGGC-3'
	Actin	NM_173979.3	102	F: 5'-GGCATCCTGACCCTCAAGTA-3'
				R: 5'- CACACGGAGCTCGTTGTAGA-3'
qPCR	BMP-15	AY572412	72	F: 5'-ATCATGCCATCATCCAGAACC-3'
				R: 5'- TAAGGGACACAGGAAGGCTGA- 3'
	GDF-9	AB058416	80	F: 5'- AGCGCCCTCACTGCTTCTATAT-3'
				R: 5'- CACACGGAGCTCGTTGTAGA-3'
	Actin	NM_173979.3	102	F: 5'-GGCATCCTGACCCTCAAGTA-3'
				R: 5'- CACACGGAGCTCGTTGTAGA-3'

2.8 Statistical analyses

Ovary harvests were analysed by One-way ANOVA, Tukey *post hoc* test, while follicle morphometric data were analysed using Pearson's Product-Moment Correlation using SPSS Inc.,(IBM Company).

Chapter 3

Results

3.1 Homogeneity of ovaries and follicle location in cortical tissue

3.1.1 Ovary weight

As fresh ovarian cortex cultures were going to be started weekly and the tissue responses compared between cultures, it was important to eliminate or minimize as many variables as possible. One component of the culture system which had the potential of varying significantly from week to week was the ovaries. The ovaries were obtained from a commercial abattoir and there was no control over the type or condition of the cows slaughtered. Information obtained from the abattoir was that most cows were approximately 2 year- old, beef- type, animals. At the time of processing the ovaries for culture, all obviously unsuitable ovaries (small and inactive, large unruptured follicles or large CLs) were rejected. All normal ovaries selected for preparation of cortex for culture were weighed and the weekly mean weights are shown in Table 3.1. Over the 10 collection days, the mean weights ranged from 6.12 to 8.30g and there were no significant differences between batches of ovaries as determined by one-way ANOVA, ($F(9,196) = 0.963$, $p = 0.473$) (See Appendix 2). At least for this parameter, batches of ovaries used to prepare cortical tissue for culture were consistent from week to week.

Table 3.1 Mean ovary weights (g) of weekly harvests of ovaries used for preparing cortical cultures.

	Harvest									
	1	2	3	4	5	6	7	8	9	10
mean	8.30	6.68	7.81	6.81	8.15	6.49	6.12	7.59	7.06	7.82
sd	4.0	2.7	2.8	3.7	4.1	2.9	3.1	2.4	4.0	3.4
n	20	22	22	20	22	22	20	20	18	22

3.1.2 Cortical location of follicles

For tissue slices to remain viable in culture, they need to be thin but contain the structure of interest. To establish the distribution of follicles in the ovary, a 3mm thick equatorial section of a typical ovary was fixed and processed for wax sectioning. The majority of all visible primordial to secondary follicles were located in the cortical region of the ovary with the highest follicle density being in the

outer 1mm of the ovary (Fig 3.1). Based on this, 1mm thick cortical slices were prepared for follicle culture.

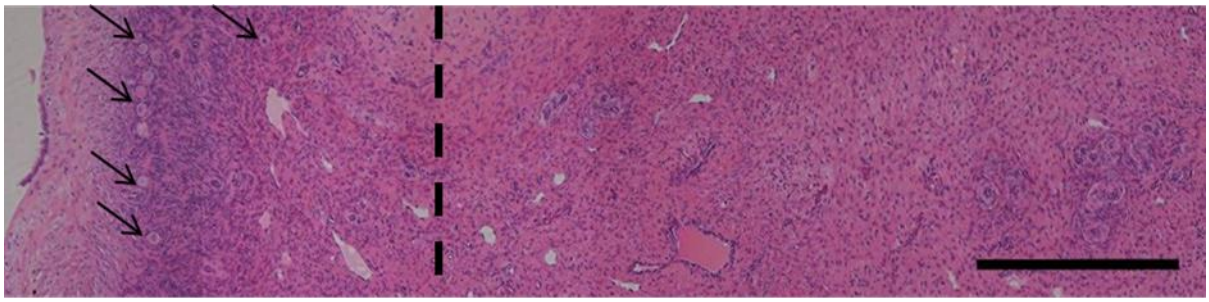

Figure 3.1 Location of primordial, primary and secondary follicles in a normal bovine ovary. A composite image of a section of bovine ovary (6µm). Arrows indicate follicles and the dotted line shows 1 mm from the surface (s), the region of cortex taken for culture. Stained with H &E. Scale bar= 0.5mm

3.1.3 Yield of slices and squares per ovary

Between one to three cortical strips were obtained from each ovary and each ovary yielded approximately 8-16 1x3x3mm cortical squares thus each batch of approximately 20 ovaries yielded about 200 squares for culture. Typically 3-5 squares were placed in a single petri dish for culture.

3.2 Cortical tissue cultures

To establish the viability of the cortical slices under the culture conditions (M199, FCS, $PGF_{2\alpha}$, PMSG at 37^{o}C, half medium changes, with PMSG & $PGF_{2\alpha}$ added to maintain starting concentrations) cortical squares were removed from cultures for fixation after 4, 7 and 10 days. This tissue was sectioned (cryostat) at 6 µm and stained with H&E. Figure 3.2 shows typical sections over the course of the culture.

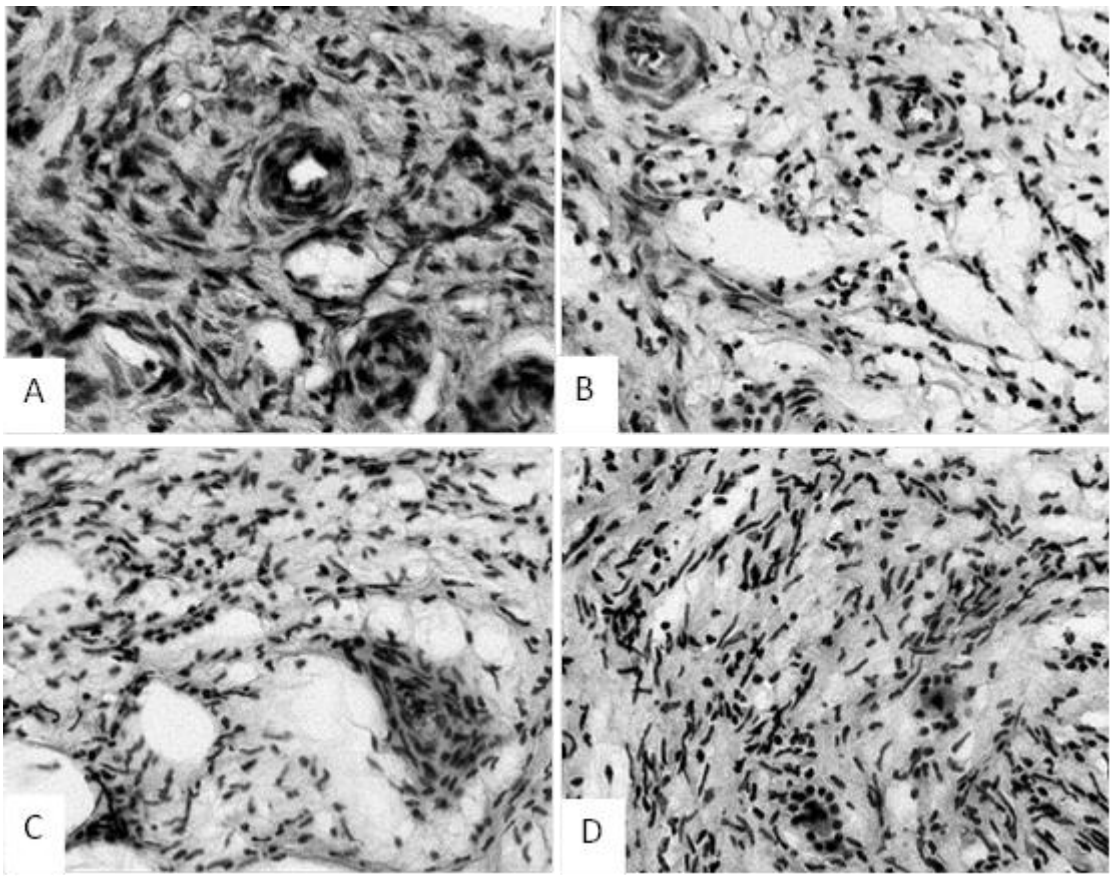

Figure 3.2 Viability of cortical tissue over 10 day culture period. Sections of cultured cortical squares supplemented with 25IU PMSG/ml showing viable tissue at (A) day 0, (B) day 4, (C) day 7 and (D) day 10 of culture.

From day 0 to day 4, (Fig 3.2 A, B) nuclei became smaller with clear boundaries and it appeared that the tissue had thinned out a little. From day 4 to day 7 the appearance of the tissue remained consistent. At day 10 of the nuclei were still well defined and there was no evidence of nuclear fragmentation or loss of tissue integrity (Fig 3.2 D).

After some early changes, the appearance and apparent viability of the tissues remained consistent to at least 10 days in culture. This suggested that the culture conditions were suitable to maintain tissue viability.

3.3 Follicle progression in cultures containing FCS

3.3.1 Quantification of follicle progression

In the ovary and under natural circumstances, as follicles become activated to proceed through the stages of folliculogenesis, they undergo various changes. Firstly the nature of the cells surrounding the oocyte changes from simple squamous to simple cuboidal to multiple layers of cells. Figure 3.3 shows progressive follicle stages. Secondly, the follicles increase in diameter as they proceed

from ~30µm (primordial) to ~50µm (primary) to ~60µm and greater (secondary). The differences in diameters between follicles at different stages of development were used to routinely classify cultured follicles.

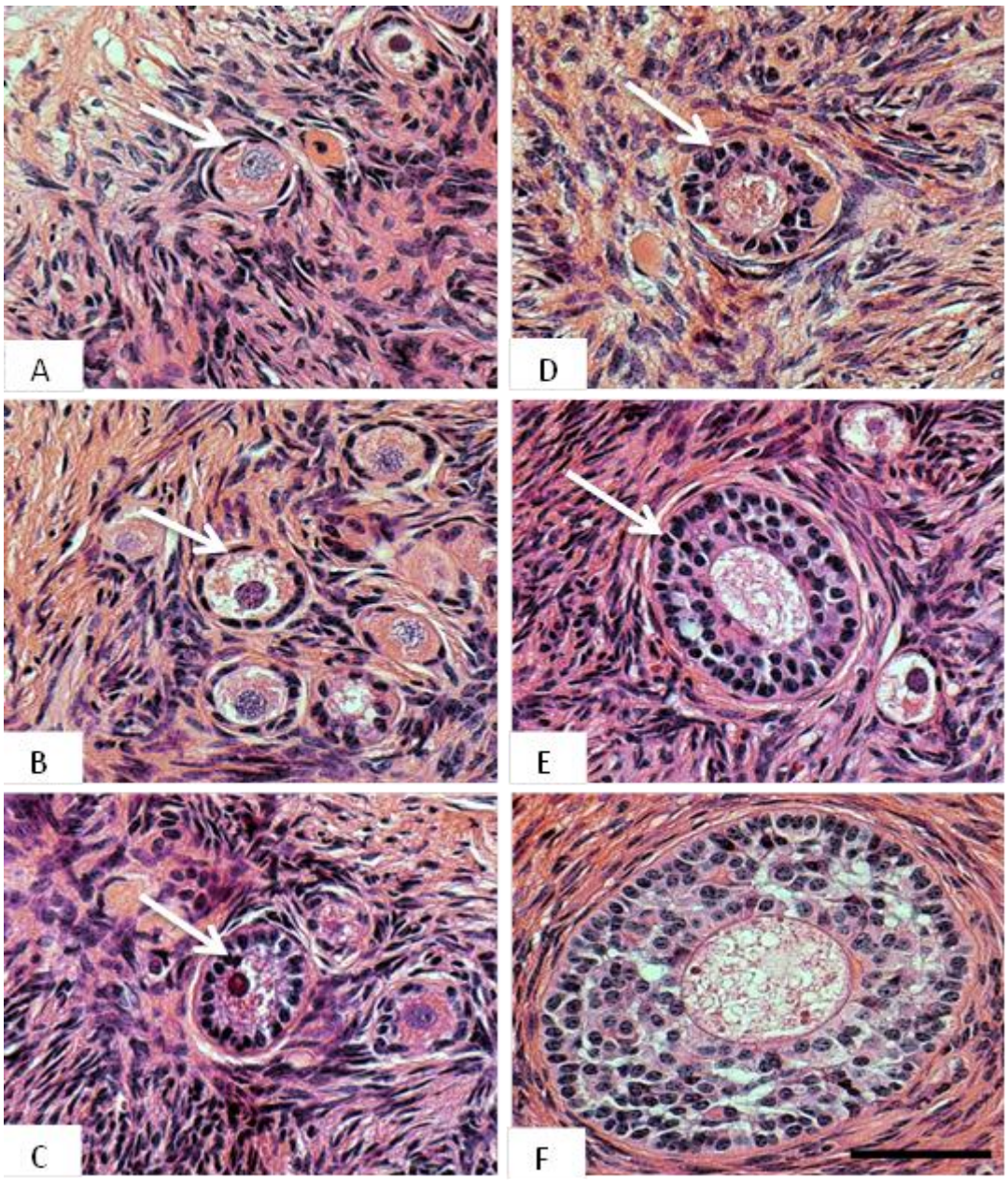

Figure 3.3 Non-cultured bovine ovarian tissue with follicles forming the base of this study. Growing follicles can be classified according to its histological and morphological features. A, B-examples of primordial follicles showing a single layer of flattened granulosa cells surrounding the oocyte, C-example of a primary follicle with single layer cuboidal cells surrounding the oocyte, D- example of an early secondary follicle showing an incomplete second layer of granulosa cells around the oocyte, E and F – examples of secondary follicles showing two or more complete layers of granulosa cells around the oocyte. Scale bar=50µm. Stained with H&E, 6µm wax section.

Follicles could be identified in cultured cortical slices (Fig.3.4) however the cellular organization was much less well defined compared to non-cultured tissue (Fig. 3.3).

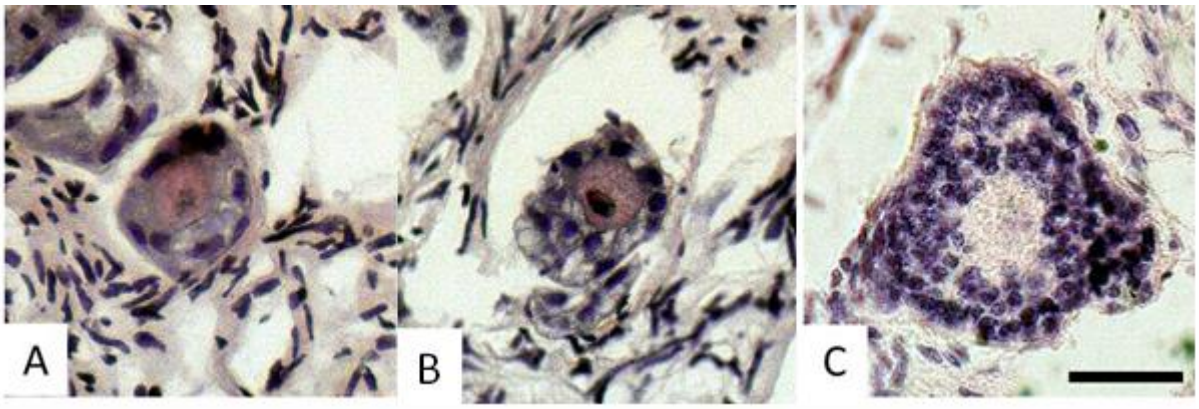

Figure 3.4 Follicles in cultured ovarian cortex. Effects of the tissue culture on the morphology of pieces of ovarian cortex from bovine ovary cultured for ten days in medium containing 50IU PMSG/ml supplemented with 10% foetal calf serum (FCS). A. Primordial follicle (F_0), B. Primary follicle (F_1) and C. Secondary follicle (F_2). All follicles appear viable with granulosa cells forming layers around a regular oocyte. Pieces of cortex were sectioned (cryostat) at 6µm and stained with H&E. Scale bar=25µm.

3.3.2 Classification of cultured follicles based on follicle diameter

The circumferences of primordial (n=80), primary (n-80), and secondary (n=79) follicles in cultured cortical slices were measured and converted to circular diameters and the results are shown in Fig.3.5. While the three follicle populations were quite distinct, there was some overlap of diameters at the extremes. To use follicle diameter as a tool to reliably categorise follicles, diameter limits needed to be established (Table 3.2).

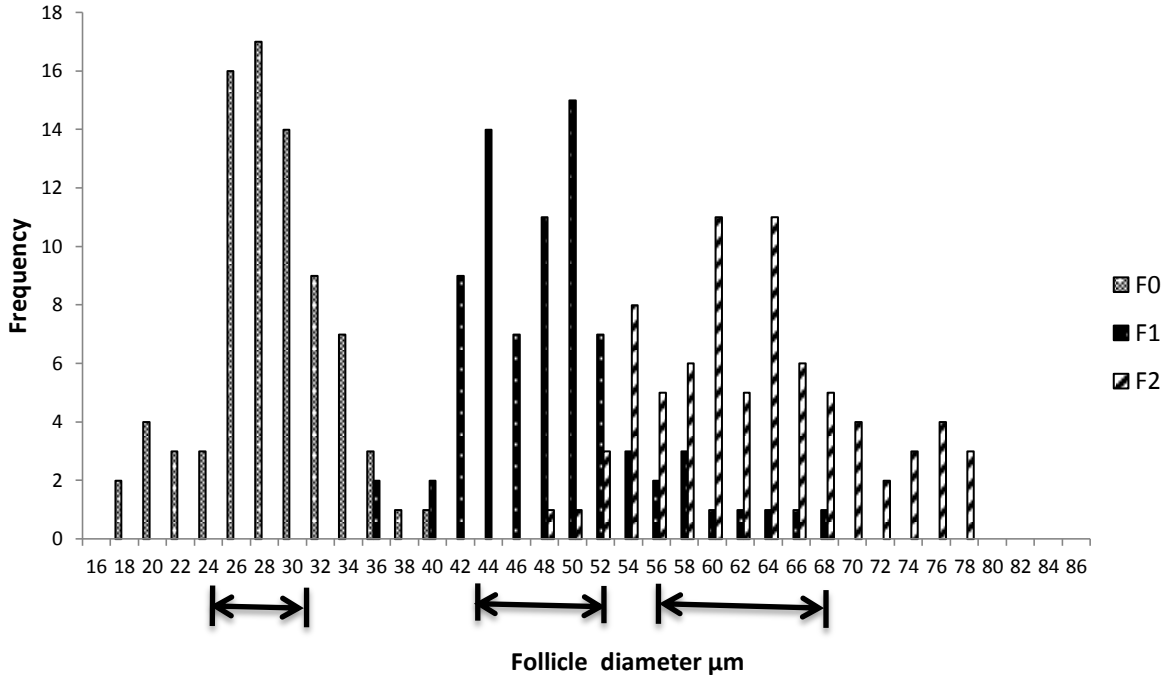

Figure 3.5 Diameter frequencies of F_0 (n=80), F_1 (n=80) and F_2 (n=79) in cultured ovarian cortex. The mean ± 0.75 sd diameter limit for each follicle stage is indicated by arrows below the axis- (See Table 3.2).

Table 3.2 Diameters of F_0, F_1 and F_2 follicles in culture. Means and 1.0 and 0.75sd limits and associated classification accuracy.

			Mean ±0.75sd		Mean ± 1.0sd	
Class	Histological feature	Mean (sd) diameter (µm)	Diameter limits (µm)	Classification accuracy (%)	Diameter limits (µm)	Classification accuracy (%)
Primordial (n=80)	Simple squamous	27.5(4.5)	24-31	100(56/56)	23-32	100(61/61)
Primary (n=80)	Simple cuboidal	47.4(6.2)	43-52	85.4(47/55)	41-53	82.4(61/74)
Secondary (n=79)	Two or more layers	62.2(7.7)	56-68	85.1(46/54)	55-70	84.3(54/64)

Follicle classification diameter limits were initially tested at mean ±1sd. When all follicle diameters were subjected to these 100% follicles between 23 and 32µm were correctly classified as F_0; 82% between 41 and 53µm correctly as F_1; 84% between 55 and 70µm correctly as F_2 follicles. The accuracy of follicle classification was improved to 100%, 85% and 85% for F_0, F_1 and F_2 respectively by reducing the diameter limits to mean ± 0.75sd (Table 3.2). Follicle diameter limits of mean ±0.75sd was applied to subsequent follicle assessments. Of the F_0, F_1 and F_2 follicles measured and

converted to circular diameters, 70%(56) F_0, fell within the mean ± (0.75sd) limits, 58%(47) within F_1 and 58%(46) within F_2 respectively.

3.3.3 Effect of gonadotropins on follicle progression in culture

3.3.3.1 Pregnant mare serum gonadotropin (PMSG)

The responsiveness of cultured follicles to gonadotropins was assessed. Duplicate cultures containing 0, 0.4, 0.625, 1.25, 2.5, 5, and 10 IU PMSG/ml and 10% FCS were set up. After 10 days in culture, the tissue sections were processed for follicle morphometric analysis. This entailed measuring the area of the first 50 follicles seen by methodically scanning through the stained sections. The diameters derived from the areas were subjected to 24-31, 43-52, and 56-68µm limits. From the total number of follicles falling with the 3 limits, the percentage of F_0, F_1 and F_2 follicles was calculated and shown in Fig. 3.6.

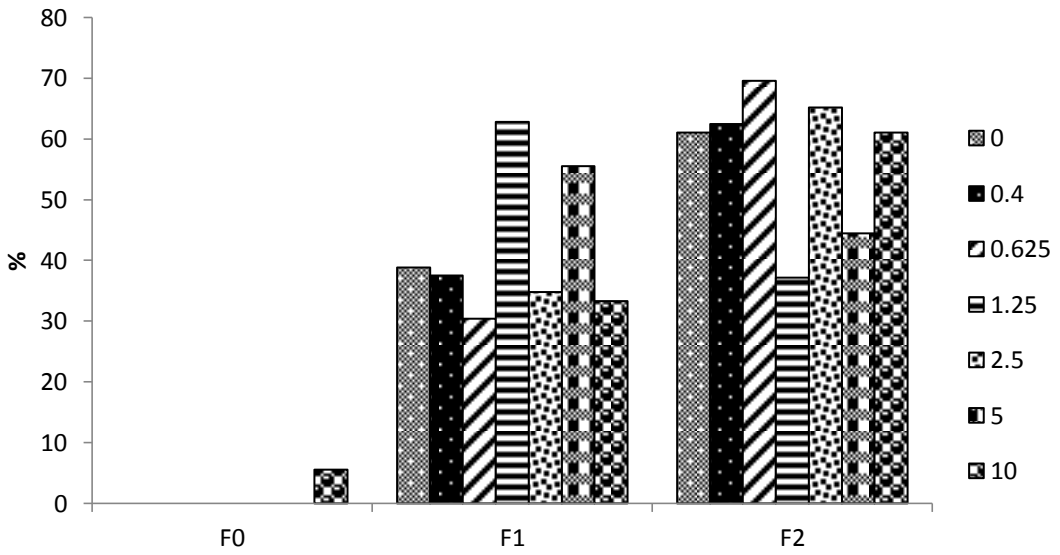

Figure 3.6 (M199 + 10% FCS). Effects of low PMSG concentrations (right of chart, IU/ml) on three classes of follicles (F_0, F_1 and F_2) at 10 day culture. These levels of PMSG failed to stimulate follicles.

Because of the lack of response with concentrations up to 10 IU PMSG/ml, the culture was repeated using 0, 12.5, 25, 50 and 100 IU PMSG/ml and the results are shown in Fig. 3.7. By increasing the maximum concentration of PMSG to 100 IU, the percentage of F_0 declined from 60 (with 50 IU) to less than approximately 10%. Concurrent with this was a decrease in the F_1 population. The F_2 population also increased with a concentrations of 100IU (PMSG). Taken together, under the

influence of higher concentrations of PMSG, follicles responded and progressed from F_0 to F_1 and from F_1 to F_2.

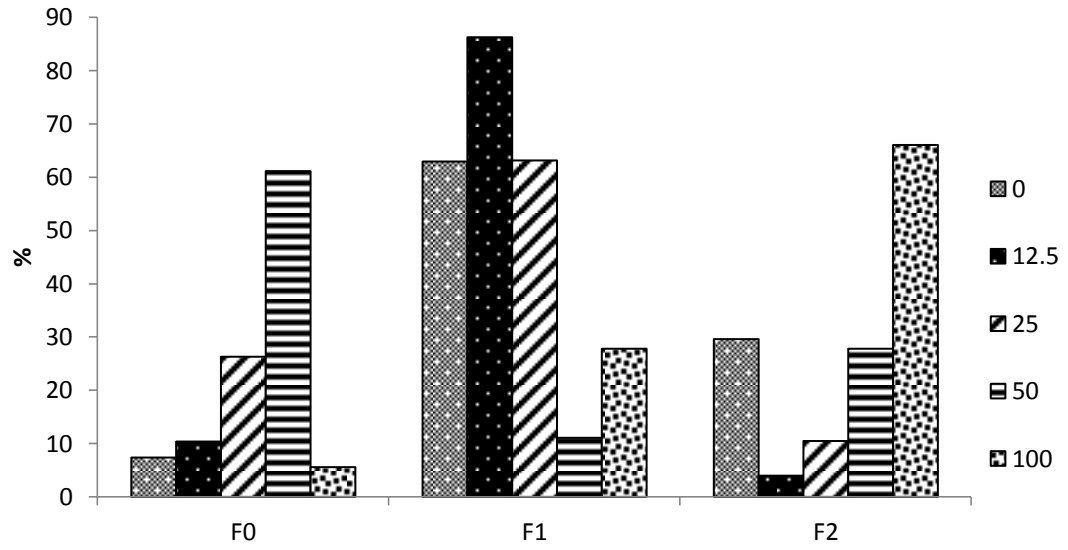

Figure 3.7 Responses of F_0, F_1 and F_2 follicles showing distinct pattern as stimulated by high PMSG concentrations ranging from 0 to 100IU/ml after 10 days in culture (M199 + 10% FCS).

3.3.3.2 Follicle stimulating hormone (FSH)

In multiple ovulation and embryo transfer (MOET) programs, various preparations of pituitary FSH have been used to stimulate the ovaries. One such product is FSH isolated from porcine pituitary glands (Folltropin®-V). Cortical cultures (4 replicate cultures) were set up containing 100ng FSH/ml and 10 % FCS. At 2, 4, 7, and 10 days, cortical squares were fixed for morphometric analysis. Control cultures contained no FSH. The distribution of F_0, F_1 and F_2 follicles with and without FSH is shown in Fig. 3.8.

FSH had a significant impact on the pattern of follicle development (Fig. 3.8). When the data from all days were combined, the presence of FSH led to a decrease in the F_0 population (30.4 vs 13%, p=0.016), no change in F_1 population and an increase in F_2 poulation (22 vs 38.8%, p=0.022) (See Appendix 3). The effect of FSH was noticeable and showed a similar trend to what was seen with high PMSG concentrations (Fig. 3.7). Overall, there is a pattern of F_0 to F_1 and F_1 to F_2 under the influence of gonadotropins.

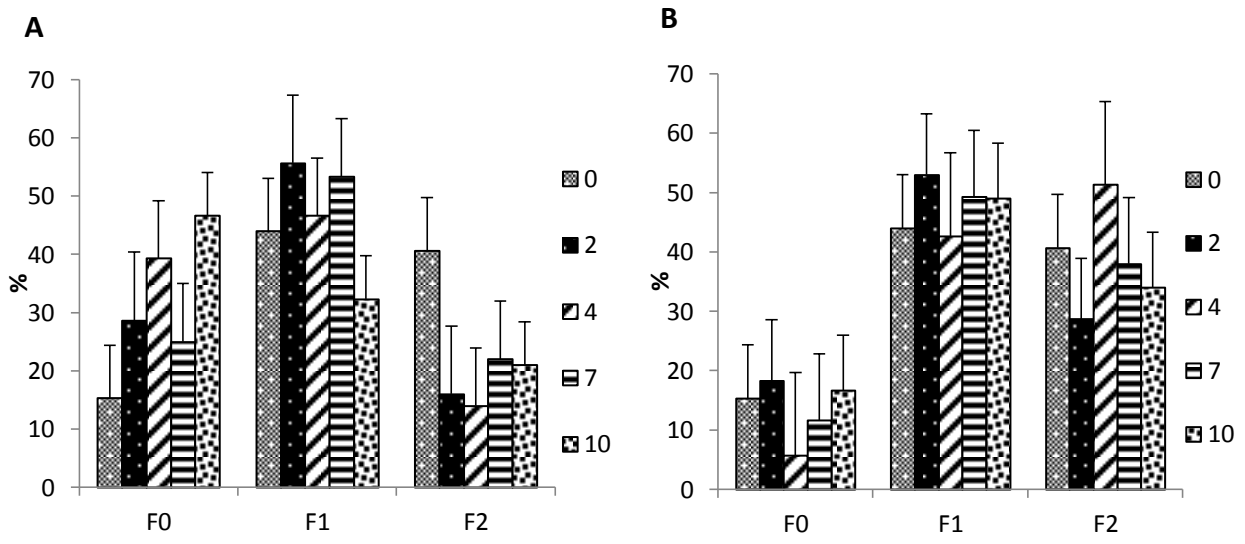

Figure 3.8 Effects of the FSH on follicle growth (F_0, F_1 and F_2) in bovine cortical slices cultured in M199+10% FCS. A. 0ng FSH/ml and B. 100ng FSH/ml. Bars 0-10 are the days in culture.

There was no clear time effect on the follicle population. Follicle progress was stimulated by day 2 after which an equilibrium was established. The data was tested by a Pearson product-moment correlation to determine the relationship between days in culture and the proportion F_0, F_1 and F_2 follicles in the presence and absence of FSH. There were no significant correlations on follicle stage and period in culture. The Pearson's correlation and P values are shown in Table 3.3.

Table 3.3 Relationship between days in culture and follicles populations. Pearson Correlation and *p* values showing no correlation between days in culture and follicle class population in the presence and absence of 100ng FSH/ml.

Treatment	Statistic	F_0	F_1	F_2
100ng FSH/ml	Correlation (r)	-0.044	0.256	-0.131
	p Value	0.944	0.678	0.834
0ng FSH/ml	Correlation (r)	0.735	-0.497	-0.422
	p Value	0.157	0.395	0.479

3.4 Follicle progression in cultures containing cow serum

Seventeen pools of cow sera (S1-S17) representing herds with potentially contrasting nutritional statuses were used to replace FCS in standard 10 day cortical cultures in presence of either 0 or 100ng FSH/ml. This experiment consisted of four replicates. As a reference, cultures containing FCS

were included in each replicate (S18). Follicles (F_0, F_1, and F_2) were assessed following the same procedures as described previously; the mean results were computed and are shown in Fig. 3.9.

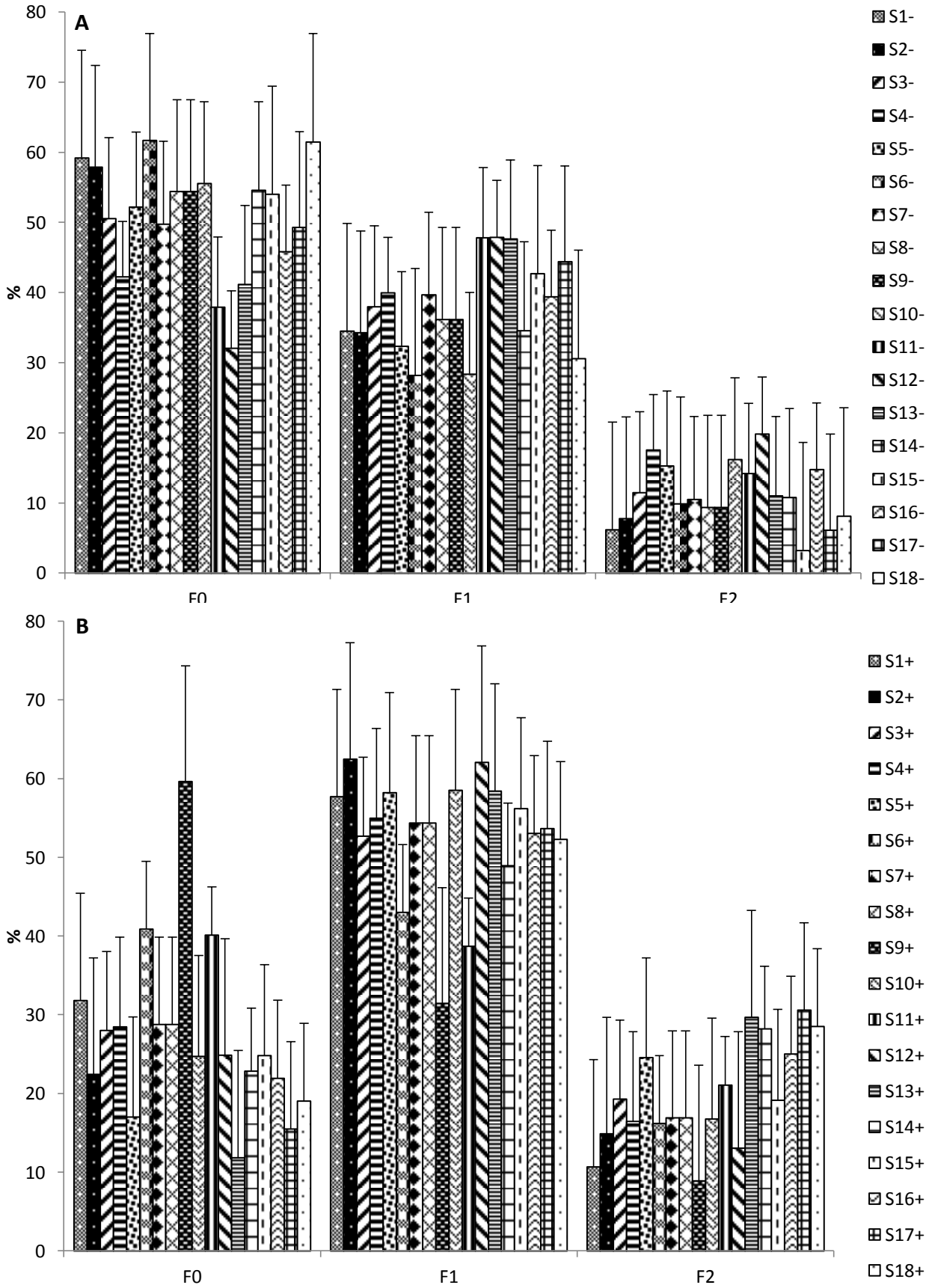

Figure 3.9 Follicle growth and development in culture medium containing cow serum after 10 days. Mean (SE) percentages of follicle populations in cultures containing 10% dairy cow sera (S1-17) in the absence (A) and presence (B) of 100ng FSH/ml. S18 is the FCS reference culture.

The pattern of follicle dynamics in the presence of FSH was in general similar to that seen in cultures with FCS (Fig. 3.8); the inclusion of 100ng FSH/ml led to a lower frequency of F_0 follicles and a higher frequency of F_2 follicles. Figure (3.10) shows the mean data for the FCS replicate references cultures (n=4) and the combined cultures (n=4) in cow sera. While the influence of FSH in the culture was consistent with the earlier data (Fig. 3.8), only the CS /F_2 difference was significant (p=0.02; ANOVA).

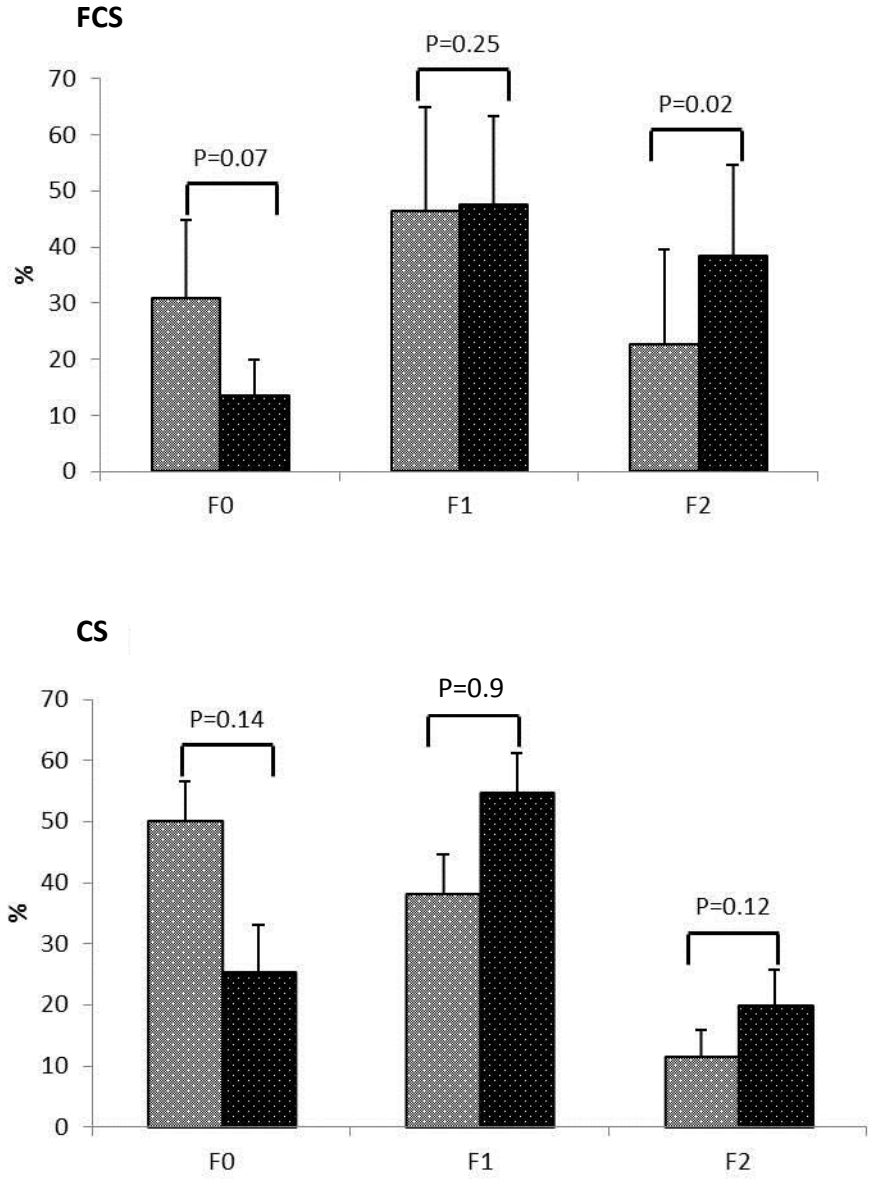

Figure 3.10 Follicle populations in cortical tissue cultured in FCS or CS for 10 days. Mean (sd) percentages of the frequency of F_0, F_1 and F_2 from 10 day cultures supplemented with either FCS or CS. Control (0ng FSH/ml) 100ng FSH/ml. One way ANOVA for the comparisons of means (+FSH vs-FSH) FCS and CS have shown F_2 in the FCS cultures was significant at *p*=0.02 (See Appendix 4)

While the overall trends agreed with the previous experiments, the noticeable variation in the follicle growth patterns between sera was likely due to the intrinsic characteristics of the dairy cow sera. To

assist with identifying trends amongst the different cow serum cultures, the data was consolidated and are shown in Fig 3.11.

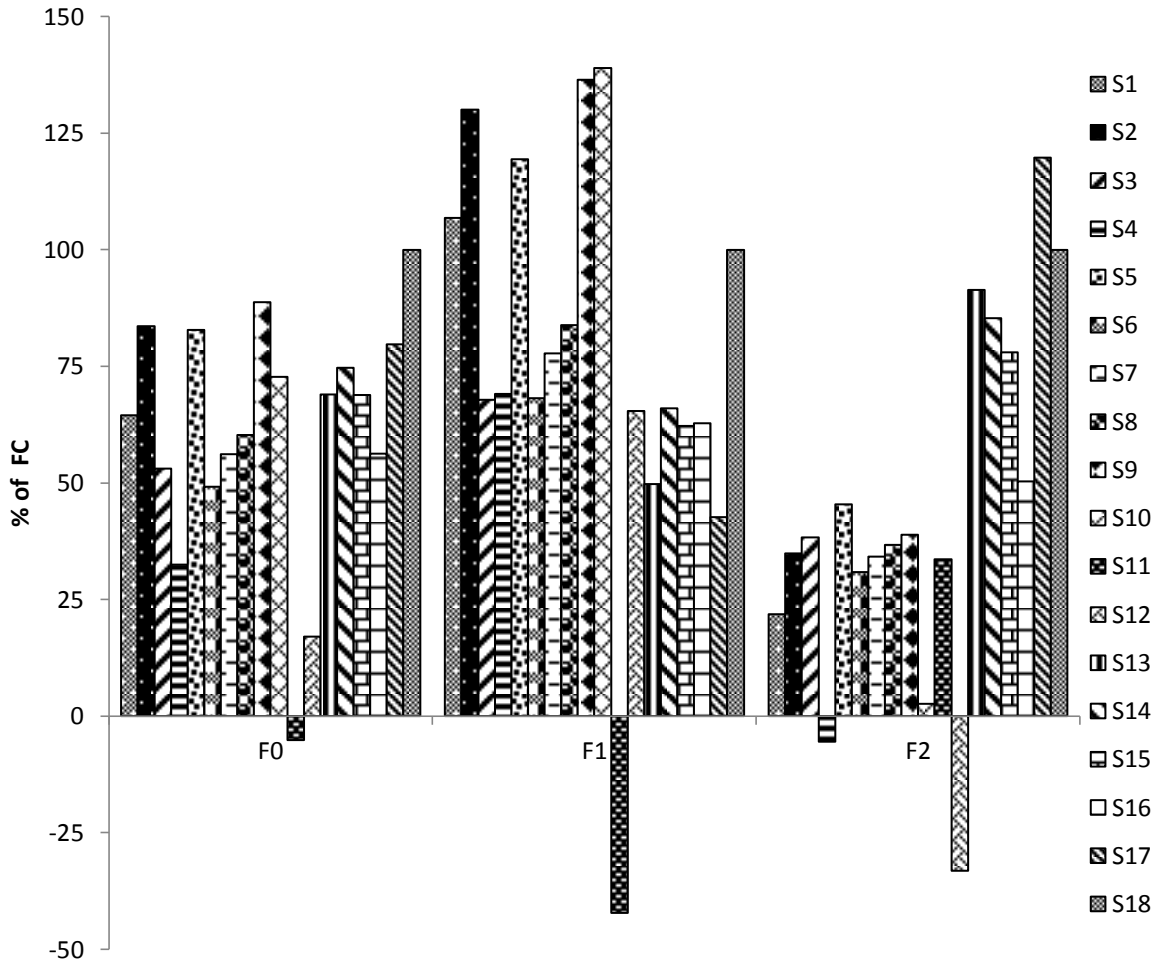

Figure 3.11 Consolidated cow serum culture performance. For each cow serum the following calculation was performed: Culture performance after 10 days relative to FCS was caclulated as

Performance = $\frac{(S_{100}-S_0)x100}{(FCS_{100}-FCS_0)}$ where S_{100}= follicle frequency serum in presence of FSH and S_0= follicle frequency in serum in the absence of FSH; FCS_{100}= follicle frequency in FCS in presence of FSH and FCS_0 = follicle frequency in FCS in the absence of FSH.

With regards to F_0, all cow sera values were lower than the FCS (S18) reference culture. Some cow sera (S2, 5, 9, 10) were marginally less than FCS whereas others (S4, S11, and S12) were considerably lower. With regards to F_1, one cow serum value (S1) was similar to FCS (S18); four were somewhat higher (S2, S5, S9, S10) and three were considerably lower (S11, S13, S17). With respect to F_2, three sera were similar but lower (S13, S14, S15), one was similar but higher (S17) than FCS (S18) while the majority were considerably lower (S1-12, S16).

3.4.1 Chemical characterisation of 17 serum pools used in cortical cultures

The 17 pools of CS and the FCS were analysed for standard cattle industry markers of nutrition status. In addition, the fertility hormone AMH was also assayed. These results are shown in Table 3.4. The normal ranges quoted by the pathology laboratory responsible for the analyses are included. To assess if there was any relationship between the chemical characteristics of the sera and the follicle dynamics displayed in the culture, numerical features of the culture were calculated and also shown in Table 3.4. These features include the nett difference in the proportions of F_0, F_1 and F_2 follicles in the presence of FSH, i.e. (S_{100}-S_0) after 10 days of culture. As an overall index of the follicle dynamics, the total of the absolute differences for the three follicle classes was also calculated (F_{Total}). The means for these parameters were -25, 17, 8 and 52 for F_0, F_1, F_2 and F_{Total} respectively. The corresponding data for FCS (S18) are also shown.

Table 3.4 Blood analytes of the seventeen sera pools used in follicle cultures and follicle culture parameters after 10 days of culture.

	Blood analytes					Follicle culture parameters			
Serum	Albumin (g/dL)	Glucose (mg/dL)	BHBA (mmol/L)	NEFA (mmol/L)	AMH (ng/ml)	F_0	F_1	F_2	F_{Total}
S1	36	0.62	0.3	0.2	0.1	-27	23	4	54
S2	38	0.99	0.3	0.2	0.13	-35	28	7	70
S3	39	0.93	0.4	0.2	0.14	-23	15	8	46
S4	37	0.58	2.3	0.3	0.09	-14	15	-1	30
S5	39	0.47	3.2	0.3	0.16	-35	26	9	70
S6	30	1.14	1.2	0.4	0.19	-21	15	6	42
S7	29	0.54	1.3	0.3	0.3	-24	17	7	48
S8	30	0.37	0.9	0.3	0.17	-26	18	7	51
S9	37	0.53	0.8	0.4	0.14	-38	30	8	76
S10	36	0.54	3.7	0.2	0.14	-31	30	1	62
S11	36	0.47	3.5	0.2	0	2	-9	7	18
S12	32	0.58	3.6	0.2	0.21	-7	14	-7	28
S13	32	0.38	3.7	0.2	0.19	-29	11	19	59
S14	30	0.33	3.1	0.2	0.08	-32	14	17	56
S15	33	0.51	3.5	0.2	0.15	-29	13	16	58
S16	32	0.34	3.2	0.2	0.09	-24	14	10	48
S17	32	0.34	3.7	0.2	0.88	-34	9	24	67
Means	34	0.47	3.47	0.25	0.19	-25	17	8	52
Sd	3.41	0.24	1.39	0.07	0.19				
FCS (S18)	29	0.09	6.00	0.2	9.14	-42	22	20	82
Normal Ranges	29-48	0-0.8	2.6-4.32	< 0.4	<1.30*				

Δ Nett difference =S_{100}-S_0, F_{Total}=Sum of absolute net differences
*GW Kay, personal communication

In the context of animal nutritional status (i.e. energy balance) serum albumin and glucose values lower than the normal range are suggestive of poor nutritional status and a negative energy balance (NEB). These two parameters suggest that none of the 17 serum pools represent animals exhibiting NEB. With respect to BHBA and NEFA, values higher than the normal range equate to NEB. Again, no serum pools used in the cultures represent animals in NEB. Low AMH values likely reflect low fertility efficiency and high AMH values indicate optimal fertility.

Regression analysis was performed for each analyte and each follicle parameter and the results are shown in Table 3.5. The analysis suggested a significant (F=0.05) correlation between AMH and F_2 however inspection of the data revealed that was largely due to a single high AMH value (S17). There was no correlation when the S17 value was excluded. A trend (F=0.09) between BHBA and F_1 appeared more real and is shown in Fig. 3.12. The lack of significant correlations between follicle growth and serum metabolites suggests that either none of the analytes quantified impact directly on follicle growth or it could also indicate that the culture methodology is not sensitive enough to detect differences. However the negative trend between BHBA and F_1 nett differences, may suggest that the higher the BHBA, the lower the nett progress from F_0 to F_1 or the higher the nett progress from F_1 to F_2.

Table 3.5 Regression analysis of blood metabolite concentrations and follicle culture parameters for the three follicle classes at d10 of culture.

Metabolite	Follicle Class	R value	F (sig.)
Albumin	F_0	0.08	0.75
	F_1	0.31	0.23
	F_2	0.34	0.16
	F_{Total}	0.17	0.51
Glucose	F_0	0.07	0.80
	F_1	0.23	0.37
	F_2	0.36	0.15
	F_{Total}	0.08	0.74
BHBA	F_0	0.27	0.27
	F_1	- 0.42	0.09**
	F_2	0.25	0.34
	F_{Total}	0.15	0.68
NEFA	F_0	0.12	0.23
	F_1	0.31	0.23
	F_2	0.20	0.45
	F_{Total}	0.10	0.05*
AMH	F_0	0.29	0.27
	F_1	0.08	0.75
	F_2	0.48	0.05*
	F_{Total}	0.3	0.23

*Significant at p= 0.05

**Significant at p<0.10

*Data skewed by single high serum value

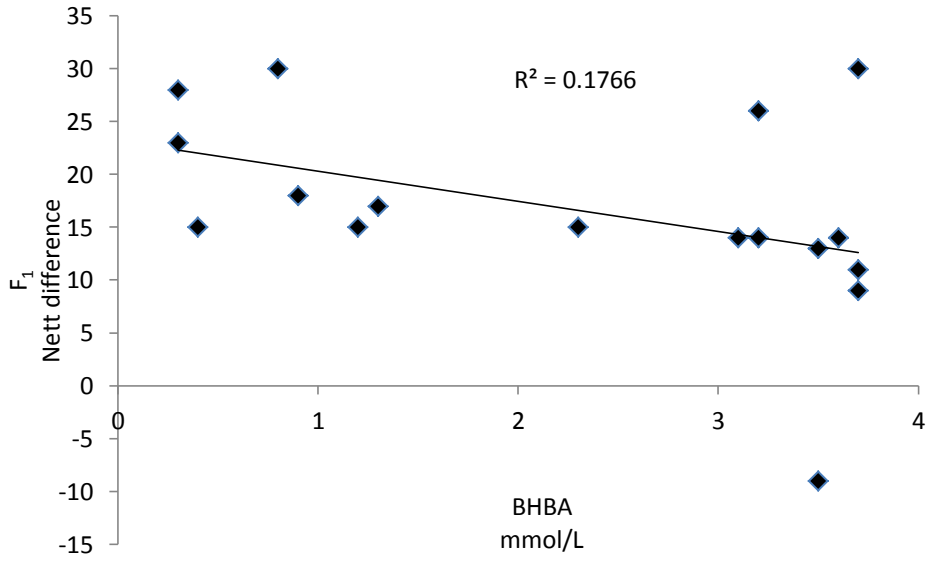

Figure 3.12 Regression of (F=0.09) between BHBA and F1 (r^2 =0.1766).

3.5 Gene expression in culture cortex

As the total RNA yield from 2-3 cultured cortical squares was below the minimum required for qPCR, tissues from cultures which performed similarly according to Fig 3.11 and Table 3.4, were pooled. As no single parameter from the follicle cultures clearly identified best or worst performing sera, the pooling of cultured tissues was based on several ways of reviewing the data. This included ranking sera for each follicle culture parameter separately (see Table 3.4) and grouping sera that on average, reflected a similar level of follicle stimulation. With regard to the culture parameters, the following were taken to reflect a positive impact: a decline in F_0, no change to F_1, increase in F_2 or a high F_{total} value. This process identified the best (High) and worst (Low) performing sera. The remaining five sera formed the third (Medium) performing sera group. The ranking of S15 was very inconsistent and was therefore not included in any group.When the culture parameters (Fig. 3.11 and Table 3.4) were taken together, three groups could be formed: High follicle growth (S1, S2, S5, S9, S14, S17), Medium follicle growth (S3, S7, S8, S10, S13) and Low follicle growth (S4, S6, S11, S12, S16). The three pools which represent high, medium and low follicle growth and their subsequent RNA yield are shown in Table 3.6.

Table 3.6 Comparative RNA yield from pooled cortical cultures.

Culture Supplements	RNA concentration (ng/ml)		
	High	Medium	Low
0ng FSH/ml	208.2	199.2	336.0
100ng FSH/ml	105.9	296.7	330.4

The A260/280 ratio of total RNA extracted ranged from 1.72 to 1.81. The RNA samples shown in Table 3.6 were checked for integrity by agarose gel electrophoresis. All the samples exhibited intact 18s and 28s RNA bands (Fig. 3.13) and were judged suitable for reverse transcription.

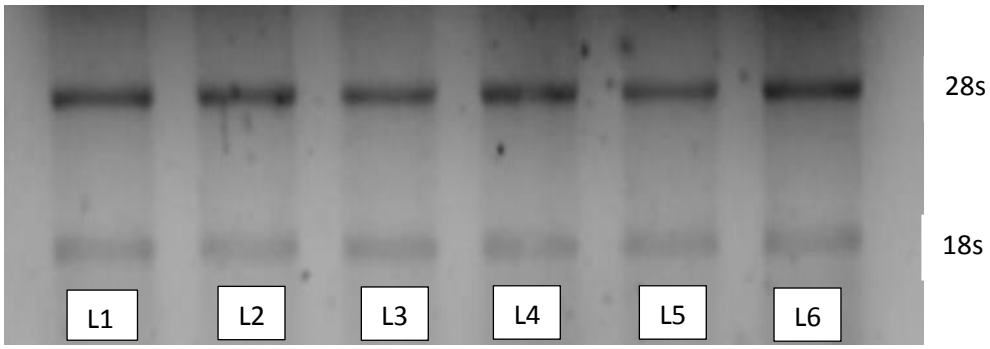

Figure 3.13 Integrity of RNA extracted from cortical tissue cultured for 10 days in M199 + 10% CS. Extracted total RNA on gel visualised under short wavelength showing intact ribosomal RNA at 28s and 18s bands respectively. L1) –FSH Low, L2) –FSH Medium, L3) –FSH High, L4) +FSH Low, L5) +FSH Medium, L6) +FSH High .

The RNA preparations were also tested to determine the presence of DNA. The DNA polymerase failed to show any amplification except in the positive control (L8, Fig 3.14).

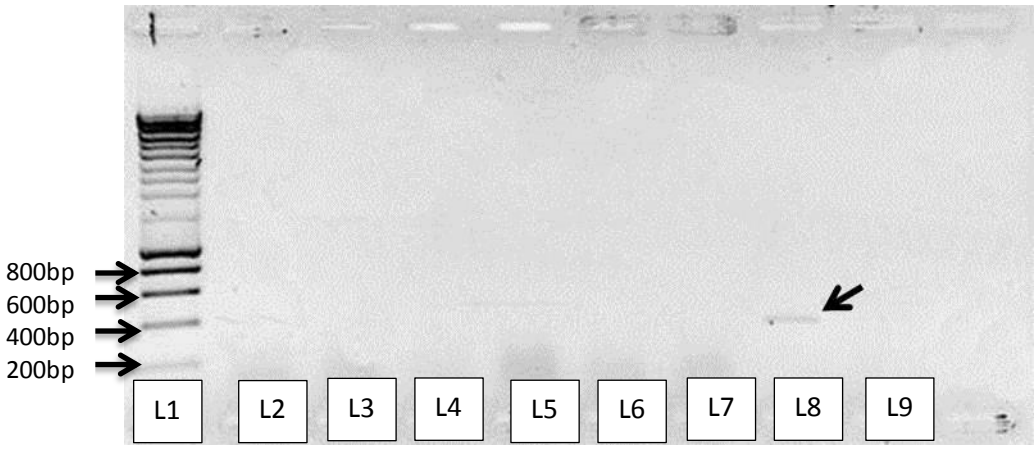

Figure 3.14 Purity of RNA extracted from cortical tissue cultured for 10 days in M199 + 10% CS. Samples indicating free of DNA contamination. (L1-Hyperladder I), (100ng FSH/ml- L2-L, L3-M, L4-H), (0ng FSH/ml-L5-L, L6-M, L7-H), (L8- Positive control (cDNA) previously validated from –FSH low RNA extracted sample. Samples in lane 8 has amplified at the expected size indicating that the PCR has performed as expected and sample in Lane 9- Negative control (H_20)- was also blank thus there was no contamination in the PCR reaction mix.

The expected PCR products were 80bp (GDF-9) and 102bp (actin). This is verified in Fig 3.15 below.

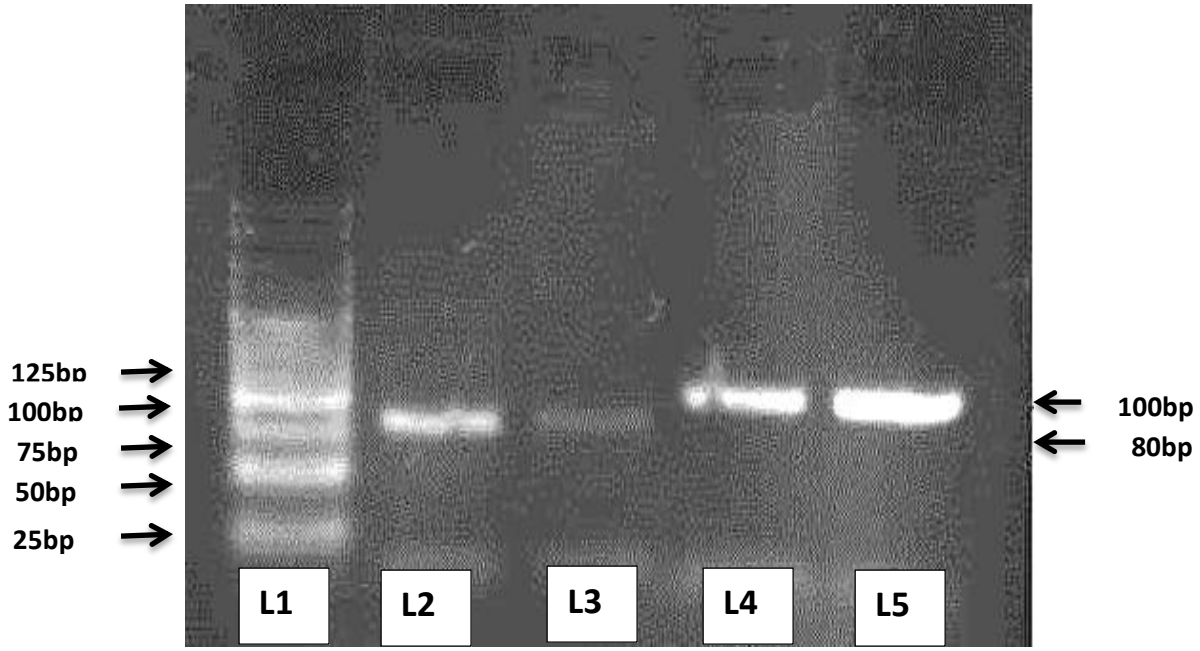

Figure 3.15 Verifying PCR products size of GDF-9 at 80bp and Actin at 102bp under the UV short wavelength light. PCR products showing the expected bands L1-Hyper Ladder V, L2 &L3-bt GDF-9 qPCR F/R (100ngFSH/ml & control) and L4 &L5- bt Actin qPCR F/R (100ngFSH/ml & control).

3.5.1 qPCR

The mean expression levels of bt GDF-9 and bt BMP-15 genes in three samples using Actin as a normalisation reference gene (0 and 100ng FSH/ml) are shown in Fig 3.16.

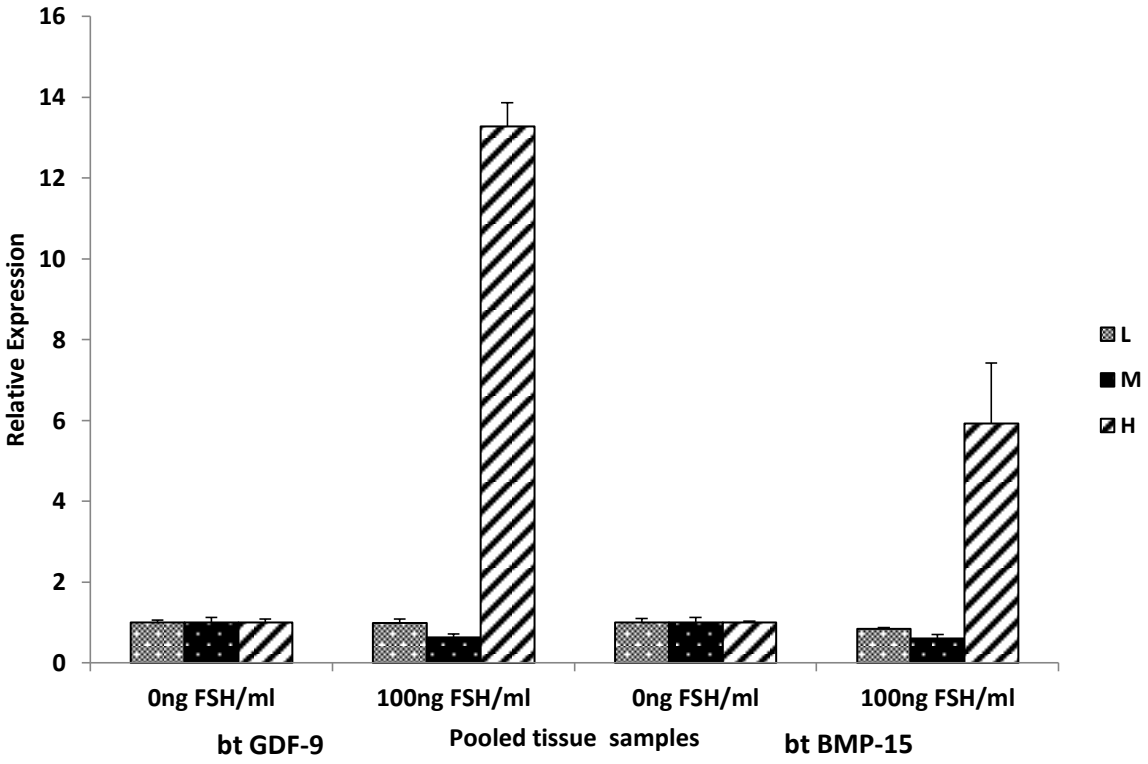

Figure 3.16 Expression of GDF-9 and BMP-15 genes in cortical tissue cultured for 10 days in M199 + 10% CS. Relative expressions of bt GDF-9 and bt BMP-15 genes in three samples of pooled cortical tissues using Bt Actin as a normalisation reference. The degree of follicle flux in the cultures were represented by: L- lowest, M- medium and H- highest. Data are presented as mean ±SEM.

Bt GDF-9 expression was nearly 14 fold higher in the culture tissue regarded as having high follicle growth. In the absence of FSH in the culture, bt GDF-9 expression levels were at basal levels. Similarly, bt BMP-15 expression was approximately 6 fold higher in the High group in the presence of FSH. Expressions of both genes were at basal levels in the Medium and Low follicle growth groups. The elevated levels of the expression of bt GDF-9 and bt BMP-15 which are both known to drive follicle progression, confirm the morphometric observations made on the cultures and the responsiveness of follicles to FSH.

Chapter 4

Discussion

This study has shown that bovine ovarian follicle growth and progression can be supported *in vitro* by culture medium containing cow serum (CS) as opposed to the commonly used foetal calf serum (FCS). Furthermore, this follicle growth and progress was found to be dependent on the source of the serum, raising the possibility that this culture system may be used as a bioassay for fertility fitness. When cortical fragments of bovine ovary were cultured in medium M199 containing 10% cow serum and FSH at physiological concentrations and assessed morphometrically, primordial follicles progressed to primary follicles and primary follicles progressed to secondary follicles (Fig. 3.10). This pattern of follicle progression was very similar to that seen in control cultures in M199 containing 10% FCS and FSH (Fig 3.8; Fig 3.10). The purpose of this study was to investigate if the metabolic profile, that is to say various plasma analytes, of cows from which the serum was sourced bore any relation to the *in vitro* follicle growth and progression of ovarian cortex cultured in the presence of these sera.

Serum was sourced from a large number (17) of different farms and/or animals to provide some variation. For the purpose of this study, the different locations and seasons were chosen merely to provide bloods with some variation in their metabolic profile chemicals for the cultures and no attempt was made to relate actual on-farm nutritional or animal conditions to the blood results. That is a complex study and was beyond the scope of the present study. When follicle progression was quantified, it was found that there was a wide variation in follicle progression between sera (Fig 3.9). Some sera performed quantitatively very similar to standard foetal calf serum, others were better while some performed considerably poorer than foetal calf serum (Fig 3.11).

Follicle progression has been observed by other researchers but most utilise a FCS-based culture medium. Foetal calf serum (usually 10%) in culture media such as M199 forms the basis of most tissue culture systems including ovarian follicle cultures and has proved suitable for various species. Using mouse ovary, (Eppig & O'Brien, 1996) found primordial follicles could be cultured to the point where the resulting matured oocytes could be fertilized *in vitro* and could give rise to live young. Using human ovarian tissue, (Hovatta *et al.*, 1999) found that primordial follicles progressed to primary and secondary stages after 1 week in culture while in baboon and cow ovarian tissues, (Fortune *et al.*, 1998) found a significant progression of growing follicles with a concomitant marked reduction in primordial follicle numbers. Similarly, Wandji *et al*.,(1996) using cow ovarian tissue, identified primordial follicle growth and progressions during 7 days in culture by an increase in diameter of primordial and primary follicles. The current study is in agreement with others in that

these various publications all demonstrate that in culture, ovarian follicles can progress through the normal developmental stages. To date, no studies have been reported using sera other than foetal calf serum or calf serum for the *in vitro* culture of ovarian tissue and all of the research quoted above utilised FCS. New born calf serum is offered by some commercial companies for the purpose of tissue culture and this has been successfully used for ovarian tissue culture (Kezele & Skinner, 2003; Nuttinck *et al.*, 1993). Cow serum has been used for rat embryo cultures but these authors found that for successful neural tube closure, methionine had to be added to the cow serum (Klein *et al.*, 1978). As FCS is the standard supplement for follicle cultures, every culture in the current study included FCS as a control.

Morphologically the ovarian cortex remained viable after being cultured for a 10 day period in medium containing gonadotropin and supplemented with 10% serum (Fig 3.2). Furthermore, follicles appeared viable with granulosa cells forming layers around a regular oocyte and of diameters consistent with growth and progression (Figs.3.4, 3.7 and 3.8). Normal follicular progression entails cytological changes in that the cell surrounding the follicles change and morphologically the diameter of the follicle increases concurrently and the latter feature was used in this study to identify follicles for quantitation. Criteria for quantitation were based on histological classification of follicles (Rodgers & Irving-Rodgers, 2010; Wandji *et al.*, 1996a) as either F_0 (one layer of flattened somatic cells around the oocyte) or F_1 (a single layer of cuboidal granulosa cells around the oocyte) or F_2 (surrounded by two or more complete layers of cuboidal granulosa cells). Their diameters were measured using image analysis software. Follicle with diameters within diameter limits of 23-32, 41-53 and 55-70µm were classed as F_0, F_1 or F_2 respectively. These diameter dimensions were based on the mean± 0.75 sd limits which had an accuracy of 100% (56/56), 85% (47/55) and 85.1% (46/54) respectively which was better than applying ±1.0sd limits (Table 3.2). The mean diameters of F_0, F_1 and F_2 follicles (Table 3.2) were similar to those reported by others for bovine (Aerts & Bols, 2010; Braw-Tal & Yossefi, 1997) and caprine follicles (Silva *et al.*, 2004).

The growth and progression of follicles has been reported to be under the control of several genes including GDF-9 (Martins *et al.*, 2008; McGrath *et al.*, 1995) and BMP-15 (Otsuka & Shimasaki, 2002). To confirm if the follicle progression seen morphometrically in the ovarian cortical cultures was being driven by these two genes, the expression of these genes as represented by the relative concentrations of their respective mRNAs was determined by qPCR. As the yield of mRNA from 1x3x3mm fragments of cultured ovarian cortical tissue was low, gene expression studies were carried out on three pools of cortical tissue cultured in 10% cow serum , representing high-, medium- and low rates of follicular growth and progression based on morphometric analysis. Relative to the reference gene (actin), GDF-9 and BMP-15 were expressed 14 fold and 6 fold, respectively, in the

high follicle growth pool (Fig 3.16). In the absence of FSH in the cultures, expression levels were not elevated (Fig 3.16). This confirmed that the *in vitro* follicle growth patterns as determined by morphometric analysis were being driven by the genes known to control this process *in vivo* and that this process was dependent on FSH.

GDF-9 and BMP-15 have been found to be expressed in oocytes (Elvin *et al.*, 1999; Erickson & Shimasaki, 2001; Hayashi *et al.*, 1999; Hosoe *et al.*, 2011; Otsuka *et al.*, 2000; Sadighi *et al.*, 2002) and surrounding follicular cells (Hosoe *et al.*, 2011) during the early (Sadighi *et al.*, 2002) and late (Bodensteiner *et al.*, 1999; Elvin *et al.*, 1999; Jaatinen *et al.*, 1999; McGrath *et al.*, 1995; McNatty *et al.*, 2001) phases of folliculogenesis in mice (Crawford & McNatty, 2012; Otsuka & Shimasaki, 2002), sheep (McNatty *et al.*, 2001) and cattle (Bodensteiner *et al.*, 1999). These two key oocyte-derived factors belong to the transforming growth factor-ß (TGF-ß) superfamily and are critical to normal ovarian function (Dong *et al.*, 1996; Otsuka *et al.*, 2011). Mutations in the BMP-15 gene in a study conducted in sheep were directly associated with a sterile phenotype, particularly in the Cambridge and Belclare breeds (Galloway *et al.*, 2000). In the current study, BMP-15 was found to increase 14 fold in cultures showing morphological progression. In another *in vitro* study in which caprine cumulus-oocyte-complexes were cultured, Kyasari *et al.*, 2012 found an approximate increase of 1.5 fold of relative expression in the presence of somatic cumulus cells. In an *in vivo* study on bovine cow and calf ovaries, Hosoe *et al.*, 2011 reported GDF-9 and BMP-15 were expressed approximately 8x higher in oocytes than in cumulus cells. It would have been preferable to quantify gene expression on tissue from all individual cultures as opposed to the pooled tissues used in this study and future studies should include at least 6 tissues squares per culture dish to ensure sufficient RNA.

The cow sera used in the cortical tissue cultures were sourced from farms and animals of potentially differing nutritional status (Canterbury, South Island West Coast and Otago) as it is known that nutritional status has an impact on fertility. Although all values for serum metabolic profile analytes fell within normal ranges (Table 3.4), there was some variation between samples (albumin: 29-39g/dL, glucose: 0.33-1.14mg/dL, BHBA: 0.3-3.7 mmol/L and NEFA: 0.2-0.4 mmol/L). Only BHBA showed a negative correlation trend with follicle progression and growth (Table 3.5), thus the higher the serum BHBA concentration, the poorer the follicle progression, suggesting that BHBA is the most sensitive of the metabolic profile analytes with respect to reproduction. Today, various blood analytes including albumin, glucose, BHBA and NEFA are used in the cattle industry as biological measures of the metabolic status of an animal on a particular level of nutrition or state of energy balance (Cerri *et al.*, 2009; Oikonomou *et al.*, 2008; Ospina *et al.*, 2010) and in animals where the energy output is greater than the input, serum concentrations of NEFA and BHBA may increase and those of glucose and albumin decline due to reserves being metabolised. The observations in this

study suggest that the internal milieu with respect to reproduction changes in advance of universal changes in the metabolic profile analytes. Blood concentrations of AMH are correlated with fertility in females including cattle (Ireland *et al.*, 2011; Monniaux *et al.*, 2010) and low AMH concentrations indicate a corresponding diminished ovarian reserve in humans (Broer *et al.*, 2011; van Rooij *et al.*, 2005) and it has become evident that serum AMH represents the best endocrine marker to assess the age-related decline of follicle number. Concentrations of AMH ranged from 0 to 0.88ng/ml in the sera used to supplement the culture media however all but one serum concentration was less than 0.3ng/ml rendering the correlation analysis unreliable. Interestingly, the concentration of AMH in the FCS in the control cultures was nearly 50x higher than the mean of the cow sera (Table 3.4). Vigier *et al*.,(1983) quantified AMH concentrations in the serum of bovine foetuses from 50 days. Compared to adult cattle, concentrations were highest on 50-100 d foetuses. Future studies may include searching specifically for animals that are on either very poor and or very good planes of nutrition and fertility to yield a wider range of metabolic profile analyte concentrations.

Despite the various metabolic levels all being normal, there were differences between the sera when incorporated into the culture medium. The failure of these metabolite levels to explain the differing culture performance of the sera suggests that none of these metabolites are directly involved with follicle growth and development and don't explain the general observation that cattle fertility is low under poor nutritional conditions. Alternatively these results could suggest that metabolites such as albumin, BHBA and NEFA and glucose are homeostatically controlled until extreme conditions occur and there may be other unknown factors that impact infertility. Thus, the culture of follicles in serum-based media maybe a very sensitive method of detecting subnormal fertility conditions. Variations in dairy cow diet and physiological state also do lead to changes in other blood parameters such as insulin (Garnsworthy *et al.*, 2008) and IGF-I (Garnsworthy *et al.*, 2008; Rhoads *et al.*, 2009) and both IGF and somatotropin have been found to play a role in regulation of follicle growth (Llewellyn *et al.*, 2007; Lucy, 2000). Quantitation of these hormones in the sera used for cortical tissue cultures was beyond the scope of the study but should be considered in follow up experiments.

In the current study, gonadotropins with follicle stimulating action were required in the culture to achieve follicle growth within the culture period of 10 days. Pregnant mare serum gonadotropin is a hormone produced by the placenta of horses and in horses has luteinising hormone (LH) - like activity. In other species such as sheep and cattle, PMSG has both LH- and FSH-like activities (Nett, 1990) and the FSH-like activity has been used to stimulate ovine and bovine ovaries to increase ovulation rate (Fortune *et al.*, 1998). In preliminary cultures with low levels of PMSG, follicle progression failed to be induced (Fig. 3.6) and it was only at a concentration of 100IU/ml did

measurable follicular progression occur within the 10-day culture period (Fig. 3.7). Purified FSH from pituitary glands is now the preferred hormone for induction of multiple ovulation in livestock. When PMSG was replaced by purified porcine FSH at the physiological concentration of 100ng/ml (Akbar *et al.*, 1974; Echternkamp *et al.*, 1994; Saha *et al.*, 2000), similar follicle progression to that with PMSG was seen (Fig. 3.10) and all subsequent cultures contained FSH at 100ng/ml. The requirement for FSH in the culture system was not only evident when the tissue was assessed morphometrically (Fig. 3.8) but also when the expression of GDF-9 and BMP-15 was quantified (Fig. 3.15).

The importance of FSH in cultures of ovarian tissues has been reported by others. FSH is an anti-apoptotic factor and helps to maintain viability of granulosa cells (Chun *et al.*, 1996) and in this way may also be beneficial in culture. Sharma *et al*.,(1999) found that FSH is an essential component for *in vitro* culture of primordial follicles from buffalo and Wu *et al*.,(2007) found that FSH is essential for *in vitro* growth and oestradiol secretion of primordial follicles from pigs. Addition of FSH to the culture media significantly increases the diameter and does have an effect on the growth of primordial, primary, and secondary follicles *in vitro* in cattle (Hulshof *et al.*, 1995; Wandji *et al.*, 1996a) and human (Wright *et al.*, 1999) tissues. Indeed, the absence of FSH didn't only prevent normal folliculogenesis but led to an abnormal process (Lunenfeld *et al.*, 1975; Wang & Greenwald, 1993). Zhou *et al*.,(1991) found that FSH leads to an increase IGF-I receptors in cultured granulosa cells suggesting some effects of FSH could be mediated via IGF-I in goat which adds weight to the suggestion above that factors such as insulin and IGF-I be assayed in future experiments. On the contrary, Braw-Tal & Yossefi, (1997) supplemented culture media with up to 100ng/ml FSH in bovine ovarian cortex cultures had noticed follicular growth starting in the 4-day of culture even in the absence of gonadotropins implying FSH is not essential in the process, and Fortune *et al*., (1998) with the same FSH concentration in cultures of cow and baboon foetal ovarian cortex showed no effect on follicle development.

In the normal ovarian cycle, progesterone must be low to allow follicle advancement to ovulation. Progesterone is secreted by the corpus luteum (CL), a temporary endocrine gland resulting from the luteinisation of the ovulatory follicle. The progesterone synthesizing capacity of the CL can be terminated by $PGF_{2\alpha}$ and in the natural oestrous cycle of cows, $PGF_{2\alpha}$ released by the uterus brings about the demise of the CL (luteolysis) which initiates the process leading to follicular growth and the next ovulation. Attempts were made to avoid corpus luteal tissue in the cortical squares used to culture, however, to ensure that no progesterone synthesis occurred *in vitro*, $PGF_{2\alpha}$ was routinely included in all cultures. The concentration of $PGF_{2\alpha}$ used (1µg/ml) has been shown by others to rapidly terminate progesterone synthesis in *in vitro* perfused luteal tissue (Watson & Maule Walker, 1978).

The bovine ovaries used in this study were sourced from 2-year old beef type cows. Over the 10 harvests ovaries were grossly consistent and showed homogeneity (Table 3.1). The individual mean ovary weights (6.12 to 8.30 g) were found to be comparable with other authors who reported a mean ovary weight of 9.3 g for 2-3 year-old cows (Foley *et al.*, 1964).

In vivo, follicles develop and grow within a 3-dimensional framework of connective tissues and various reports have shown that their growth is best in a 3-D format. Loret de Mola *et al*.,(2004) found that collagen-treated/collagen gel encapsulation of mouse follicles led to large follicles after a 14-day culture period size. Earlier studies on bovine (Itoh *et al.*, 2002) and human, (Hovatta *et al.*, 1999) tissues found this method possible to culture primordial follicles, with many of them reaching secondary stage after ~ 4 weeks of culture. One of the constraints of 3-D culture is the ability to supply nutrients and O_2 to the cells. This is more easily achieved in 2-D cultures where the medium is in direct contact with all the cultured cells but follicles grown in 2-D lose their shape and flatten out (Hartshorne, 1997) and became atypical. In the current study, follicles were cultured within their cortical framework which consisted of slicing off the cortex in a controlled manner to yield 1mm thick slices. Through the culture period there was no evidence of nuclear breakdown as the nuclei in the cultured tissue were intact and round with a smooth boundary (Fig. 3.2) and in addition, mRNA could be extracted from the cultured tissues. To maximise the survival of the cortex, the ovaries were processed following set protocols, transported on ice, and placed in culture as soon as possible (approximately 5h elapsed from the time of slaughtered until the time the culture placed in the incubator). At day 10 of culture the nuclei were still well defined and there was no evidence of nuclear fragmentation or loss of tissue integrity and it is likely that the apparent thinning out of the tissue in culture contributed to its viability (Fig 3.2 D). Messenger RNA is known to be unstable (Ross, 1995) and in mice, the average half-life of mRNA is only 7 hours (Sharova *et al.*, 2009). In the current study, intact good quality message was able to be extracted from cortex which had been in culture for 10 days (Fig 3.13), further suggesting that the culture conditions employed promoted tissue viability and physiological responsiveness. Thinning out of slice cultures has been seen by others during the first few weeks of culture and is seen as a positive effect in that cells are more visible and accessible for physiological study (Barker & Goslin, 1998). Comparing the format for organ cultures, Hovatta *et al*.,(1999) demonstrated that survival of ovarian follicles was significantly better if cultured within tissue slices than it was among partially isolated follicles. This is similar to work by Wandji *et al.*, (1997) which indicated that follicles in tissue mechanically isolated fragments survived better in culture than enzymatically isolated follicles. While Hovatta *et al*., (1997) found more follicles on ovarian cortex slices that appeared to have an advantage over enzymatic and mechanical isolation processes. The ovarian cortical squares used in this study contained the primordial follicle population located in a thin, relatively avascular layer outermost in the ovarian cortex was

successfully cultured under *in vitro* conditions and compares well with studies conducted by Martins *et al*.,(2005); Matos *et al*., (2011); Silva *et al*.,(2006) who showed that follicles embedded in cortical tissue respond to hormones and that follicles respond by developmental progression while primordial follicles reduce, as found in cow (Wandji *et al.*, 1996a) , goat (Braw-Tal & Yossefi, 1997) and baboon (Fortune *et al.*, 1998).

In conclusion, an *in vitro* culture system for bovine ovarian cortex has been established and the growth and progression of the ovarian follicles has been shown to be influenced by the origin of the serum used to supplement the culture medium. The concentration of one of the metabolic profile analytes, namely BHBA, showed a trend with follicle growth suggesting that the variation seen in follicle growth reflected the energy balance and or nutritional status of the animals from which the serum was sourced. This agrees with the general observation that poor nutrition adversely affects fertility in cattle. That none of the other components of the metabolic profile (albumin, glucose and NEFA) showed any correlation with the *in vitro* growth of the follicles suggests that the culture system is more sensitive than blood metabolites as markers of the internal milieu. An *in vitro* culture system is a practical approach for follicle growth bioassay to assess the effect of the internal milieu with respect to fertility prospects. The results of the cow serum on the relative expression of two target genes GDF-9 and BMP-15 and the profound results of cow serum in the ovarian cortical pieces in cultures support the hypothesis that exposure of ovarian oocytes to unfavourable physiological events during follicle development from primordial to pre-ovulatory stage may result in the ovulation of defective oocytes after the insult (Britt, 1992; Fair, 2010).

Another aspect of this work is that it contributes to ovarian cortex biotechnology. In general terms, the idea of improving the follicle cultures to the point where the oocytes may be fertilised has a promising future. This could super accelerate the genetic gain by culturing foetal follicles to ovulation to produce offspring and therefore skipping a generation. However, to date only cultures of mouse follicles have produced live offspring (O'Brien *et al.*, 2003) but this capability could be of great benefit to the livestock industry. Another area of research receiving a lot of attention at present is the preservation of fertility in special cases such as in women undergoing chemotherapy for cancer. Chemotherapy would normally render the women infertile so techniques are now being developed to preserve fertility by removing the ovaries prior to treatment and then either culturing follicles for *in vitro* fertilisation (Cha *et al.*, 1991) or surgical return of ovarian tissue post-chemotherapy (Gosden, 2008).

Appendices

Appendix 1 – Reverse transcription and quantitative PCR

1. Primers

1.1 Sequences

a) Template cDNA

BMP-15 377bp Forward/Reverse (5'- CAAGCAGGCAGTATTGCATCTGAA-3' and 5'- TCACCTACATGTGCAGGACTGGGC-3'),

GDF-9 401bp Forward/Reverse (5'- GAAGCTGCTGAGGGTGTAAGATT-3' and 5'- AAGCAATTGAGCCATCAGGC-3')

Actin 102bp Forward/Reverse (5'-GGCATCCTGACCCTCAAGTA-3' and 5'- CACACGGAGCTCGTTGTAGA-3')

b) qPCR cDNA

BMP-15 72bp Forward/Reverse (5'-ATCATGCCATCATCCAGAACC-3' and 5'- TAAGGGACACAGGAAGGCTGA- 3'

GDF-9 80bp Forward/Reverse (5'- AGCGCCCTCACTGCTTCTATAT-3' and 5'- TTCCTTTTAGGGTGGAGGGAA-3')

Actin 102bp Forward/Reverse (5'-GGCATCCTGACCCTCAAGTA-3' and 5'- CACACGGAGCTCGTTGTAGA-3')

1.2 Primer preparation

Synthesis and dilution

Lyophilized oligonucleotides *Bos Taurus* (bt) Bone Morphogenetic Protein-15 (BMP-15) Forward/Reverse, *Bos Taurus* (bt) Growth Differentiation Factor-15 (GDF-9) Forward/Reverse, bt BMP-15 qPCR Forward/Reverse, bt GDF-9 qPCR Forward/Reverse and bt Actin Forward/Reverse (Integrated DNA Technologies, Leuven, Belgium) were centrifuged at max speed for 3 minutes prior to opening the tube for resuspension. Tris-EDTA (10mM Tris pH 8.0; 0.1mM EDTA; pH 8.0) was used for resuspending dry oligonucleotides to a storage stock of 100µM by adding 1µL volumes of TE at ten times the number of nanomoles. After addition, the tubes were vortexed for 10-15 minutes to resuspend completely. The stock tubes were briefly centrifuged and then 10µM working stocks created (e.g. an oligonucleotide from bt BMP-15 F sequence has oligo amount of 24.1nmol, to make the storage stock of 100µM add 241µL of TE and then take 10µL of this to a new tube and add 90µL of sterile water to make a working stock of 10µM).

2 RNA extraction

2.1 Extraction Procedure

Tissue samples (cortical square pieces) at the end of 10 days in culture were used for gene expression studies. To minimise RNA damage, samples were directly snap frozen in liquid nitrogen and kept in a -80^{o}C freezer until use. Tissue sample grinding was performed using a mortar and pestle/liquid nitrogen. Thereafter, ground samples were transferred into 1.5 ml autoclaved tubes and 1 ml Trizol reagent (Invitrogen Co, Auckland, New Zealand) added to each sample. After five minutes incubation at room temperature, 0.2 ml chloroform was added to the samples, incubated at room temperature for two minutes and then centrifuged at 12, 000× g for 15 minutes at 4°C. The top aqueous layer containing RNA was carefully removed by pipetting to a labelled 1.5 ml tube with 0.5 ml isopropanol added. Samples were allowed to incubate for ten minutes at room temperature before being centrifuged at 12, 000× g for ten minutes at 4°C. The supernatant was discarded and the white-cream like gel RNA pellet was washed with 1 ml of 75% ethanol by centrifugation at 7,500× g for five minutes at 4°C. The supernatant was removed and the RNA pellets air dried for 15 minutes to remove excess liquid in the tubes. Pellets were then dissolved in 50 µl of RNase free water and incubated in a heat block for 10 minutes at 55-65°C to completely dissolve the pellet.

2.2 RNA quality and quantity

a) 260/280 ratio

To remove any DNA contamination resulting from the extraction method, the Turbo DNA-free (Life technologies, Auckland, New Zealand) procedure was followed as per manufacturer's instructions. After DNase treatment the integrity of the RNA was assessed using agarose gel electrophoresis. Extracted RNA was quantified by spectroscopy using a NanoDrop 1000c spectrometer (Thermo Scientific, USA) based on ultraviolet (UV) absorbance of DNA at 260 nm. A 260/280 ratio of >1.8 indicates pure RNA.

b) Agarose gel

The integrity of the RNA was assessed using agarose gel electrophoresis. Agarose gels (1%) were made using 0.35g agarose and 35 ml 1× Tris-borate-EDTA (TBE) buffer solution. Agarose was dissolved by microwaving the solution for 35 seconds until clear and then was left to cool slightly before 2 µl sybersafe dye (Invitrogen, Auckland, New Zealand) was added. The gel was poured into an RNase free gel boat and left to set for 20 minutes. From each extracted RNA, a total of 1500ng, 2µl dye (5 × loading buffer blue) (Bioline, Total Lab Systems, New Zealand) and RNase free water to a final volume of 10µl were added to a new PCR tube and heated at 65°C for 10 minutes, and allow to cool for 1 min on ice before being loaded in the wells. Electrophoresis was run at 85volts for 45

minutes. The gel was then visualised under short wavelength UV light (245 nm) using Uvitec Fire Reader (Uvitec Ltd., Cambridge, UK).

c) DNA contamination (using PCR + Template primers)

The end-point PCR was set up using DNA polymerase and RNA as template to check the presence of contaminating DNA. Each individual PCR reaction contained the following (Table A).

Table A. PCR reaction mix for checking DNA contamination in RNA via end-point PCR.

Reagent	**Volume**
Total RNA	1µL
10x Taq Buffer	2µL
dNTP mix (10µM each)*	0.4µL
Forward Primer (10µM)	0.4µL
Reverse Primer (10µM)	0.4µL
Taq Polymerase*	0.1µL
Sterile dH_2O	15.7µL
Total	**20µL**

*Manufacturer details

The PCR conditions were comprised of 1) an initial denaturation step at 95°C for 3min, 2) 95°C for 30sec, and 3) annealing step at 58°C for 20sec, 4) elongation step 72°C for 30sec, 5) steps 2 to 4 for 30 cycles, 72°C for 1min then, 6) hold 10°C indefinitely. To check for the presence of contaminating DNA an end-point PCR was set up using DNA polymerase and the prepared RNA as a template. In theory the DNA polymerase should not be able to amplify the RNA target and the resultant agarose gel should show no bands. PCR products were analyzed using agarose gel electrophoresis and visualized with (Fire Reader, Uvitec, Cambridge, UK). Primer sets GDF-9 and BMP-15 were expected below 400bp in hyperladder 1 marker.

3 Template cDNA synthesis by reverse transcription

Total RNA extracted contains the gene specific transcripts known as mRNA. To investigate the gene expression levels mRNA was converted into *cDNA*. This was achieved via an enzymatic reaction using the reverse transcriptase enzyme. The *cDNA* was synthesised using Blueprint RT reagent kit (Takara Bio, Inc., Japan). The following reaction was setup for the generation of *cDNA* (Table B).

Table B. Reaction mix used for *cDNA* synthesis.

Reagent	Volume
5X BluePrint Buffer	2µl
BluePrint Enzyme Mix	0.5µl
Oligo dT Primer (50µM)	0.5µl
Total RNA (500ng)	Varies according to RNA concentration
Sterile dH_2O	Varies depending on RNA concentration
Total	10µL

Up to 500ng of total RNA was reverse transcribed in a 10µL reaction. The reaction was performed in a thermocycler under the following condition: 37°C for 15min (Reverse transcription) 85°C for 5sec (Inactivation of the reverse transcriptase with heat treatment) then held at 10°C indefinitely. The reaction was diluted 10 fold by adding 90µL of sterile dH_2O. The total conversion yield of *cDNA* was approximately 500ng.

Following Herath *et al*.,(2004) and Hosoe *et al.,*(2011) two sets of primers *Bos Taurus* (bt) Bone Morphogenetic Protein- 15 (BMP-15) Forward/Reverse (5'-CAAGCAGGCAGTATTGCATCTGAA-3' and 5'- TCACCTACATGTGCAGGACTGGGC-3'), and *Bos Taurus* (bt) Growth Differentiation Factor- 9 (GDF-9) Forward/Reverse (5'- GAAGCTGCTGAGGGTGTAAGATT-3' and 5'-AAGCAATTGAGCCATCAGGC-3') that generate 377- and 401-bp fragments respectively were used. The GenBank accession numbers of bt BMP-15 and GDF-9 for PCR analysis are AY572412 and AB058416. The reference gene bt Actin: 5'-GGCATCCTGACCCTCAAGTA-3' and 5'- CACACGGAGCTCGTTGTAGA-3') generated 102-bp fragments. The GenBank accession number of bt Actin is NM_173979.3 (Cock-Rada *et al.*, 2012). All primers were commercially synthesized (Integrated DNA Technologies, Belgium). PCR products were analysed using 3.5% agarose gel electrophoresis (85volts at 35 minutes, Fig. 3.15).

4 qPCR

Precision pipetting of reagents were carried out by ep*Motion*® 5070 with integrated PC and epBlue™ Version 10 (Hamburg, Germany) reaction mix are shown in (Table C). The qPCR analyses for the gene expression of bt GDF-9, bt BMP-15 and bt Actin in cultured cortical square tissues were carried out by SYBR assay as previously reported (Hayashi *et al.*, 2010; Hosoe *et al.*, 2011; Ushizawa *et al.*, 2007). The following were the thermal cycling conditions and included initial sample incubation at 50^{o} for 2 min and 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 15 sec and at 60^{o}C for 1 min. The cycle threshold values (ΔCt) indicated the fold change of the target gene in each sample, and the sequence of the target gene was determined in real time using an Eco™ Real-Time PCR System Ver. 4.0, Illumina® (San Diego, CA, USA). Standard curves were generated for each gene by serial dilution of containing bt BMP-15, bt GDF-9, and bt Actin *cDNAs* to quantify the amplified products. Since this

was an exploratory experiment to validate the tissue culture results primer efficiency test was not performed and was assumed to be 100%. Real-time qPCR was performed using primers (Bt BMP15: 5'-ATCATGCCATCATCCAGAACC-3' and 5'- TAAGGGACACAGGAAGGCTGA- 3', bt GDF-9: 5'-AGCGCCCTCACTGCTTCTATAT-3' and 5'- TTCCTTTTAGGGTGGAGGGAA-3', bt Actin: 5'-GGCATCCTGACCCTCAAGTA-3' and 5'- CACACGGAGCTCGTTGTAGA-3') that generate 72-, 80- and 102-bp fragments. The GenBank accession number of bt Actin is NM_173979.3 (Cock-Rada *et al.*, 2012). Each reaction was carried out in duplicate. The relative expression level of each target gene was calculated by the relative standard curve and ΔΔCt methods using EcoStudy software, Illumina® (San Diego, CA, USA).

Table C. Reaction mix used for qPCR

Reagent	Volume
2xSYBR	5µl
Primer Forward (10uM)	0.2µl
Primer Reverse (10uM)	0.2µl
PCR grade water	0.6µl
Diluted cDNA template	4µl

Appendix 2 – Ovary weights statistics

ANOVA

weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	106.370	9	11.819	.963	.473
Within Groups	2408.581	196	12.289		
Total	2514.952	205			

Appendix 3- One way ANOVA for the comparison of means (+FSH vs –FSH) FCS

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	5	152	30.4	139.8
Column 2	5	65	13	26.5

ANOVA

F_0

	Sum of Square	df	Mean Square	F	Sig.	F Critical
Between Groups	756.900	1	756.900	9.103	0.017*	5.318
Within Groups	665.200	8	83.150			
Total	1422.100	9				

*Significant at F- (1, 8) = 9.103, p=0.02

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	5	225	45	72.5
Column 2	5	227	45.4	16.3

ANOVA

F_1

	Sum of Square	df	Mean Square	F	Sig.	F Critical
Between Groups	0.400	1	0.400	0.009	0.927	5.318
Within Groups	355.200	8	44.400			
Total	355.600	9				

UMMARY

Groups	Count	Sum	Average	Variance
Column 1	5	225	45	72.5
Column 2	5	227	45.4	16.3

ANOVA

F_2

	Sum of Square	df	Mean Square	F	Sig.
Between Groups	705.600	1	705.600	7.897	0.023*
Within Groups	714.800	8	89.350		
Total	1422.100	9			

* Significant at F- (1, 8)= 7.897, p=0.023

Appendix 4- one way ANOVA for the comparisons of means (+FSH vs-FSH) FCS and CS

CS

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	4.000	202.000	50.500	733.667
Column 2	4.000	101.000	25.250	188.250

ANOVA

F_0

	Sum of Square	df	Mean Square	F	Sig.	F Critical
Between Groups	1275.125	1	1275.125	2.766	0.147	5.987
Within Groups	2765.750	6	460.958			
Total	4040.875	7				

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	4	152	38	366
Column 2	4	220	55	80.667

ANOVA

F_1

	Sum of Square	df	Mean Square	F	Sig.	F Critical
Between Groups	0.400	1	0.400	0.009	0.927	5.318
Within Groups	355.200	8	44.400			
Total	355.600	9				

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	4	46	11.5	56.333
Column 2	4	79	19.75	28.9167

ANOVA

F_2

	Sum of Square	df	Mean Square	F	Sig.	F Critical
Between Groups	136.125	1	136.125	3.194	0.124	5.987
Within Groups	255.750	8	42.625			
Total	391.875	9				

FCS

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	4	246	61.5	1009.67
Column 2	4	76	19	492.667

ANOVA

F_0

	Sum of Square	df	Mean Square	F	Sig.	F Critical
Between Groups	3612.500	1	3612.500	4.809	0.071	5.987
Within Groups	4507.000	6	751.167			
Total	8119.600	7				

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	4	122	30.5	1031
Column 2	4	209	52.25	118.25

ANOVA

F_1

	Sum of Square	df	Mean Square	F	Sig.	F Critical
Between Groups	946.125	1	946.125	1.647	0.247	5.987
Within Groups	3447.75	6	574.625			
Total	4393.88	7				

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	4	32	8	36.667
Column 2	4	115	28.75	153.583

ANOVA

F_2

	Sum of Square	df	Mean Square	F	Sig.	F Critical
Between Groups	861.125	1	861.125	9.052	0.024	5.987
Within Groups	570.75	6	95.125			
Total	1431.88	7				

* Significant at F- (1, 6)= 9.053, p=0.024

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